(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 June 2004 (10.06.2004)

PCT

(10) International Publication Number
WO 2004/048404 A2

(51) International Patent Classification⁷: **C07K 14/22**

(21) International Application Number: PCT/IB2003/006320

(22) International Filing Date: 21 November 2003 (21.11.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0227346.4 22 November 2002 (22.11.2002) GB

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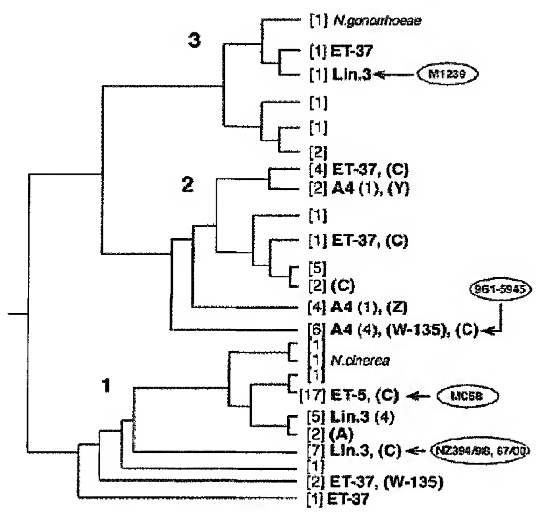
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

[Continued on next page]

(54) Title: MULTIPLE VARIANTS OF MENINGOCOCCAL PROTEIN NMB1870



(57) **Abstract:** Meningococcal protein NMB 1870 has been described in the prior art. The inventors have found that NMB 1870 is an effective antigen for eliciting anti-meningococcal antibody responses, and that it is expressed across all meningococcal serogroups. Forty-two different NMB 1870 sequences have been identified, and these group into three variants. Serum raised against a given variant is bactericidal within the same variant group, but is not active against strains which express one of the other two variants i.e. there is intra-variant cross-protection, but not inter-variant cross-protection. For maximum cross-strain efficacy, therefore, the invention uses mixtures comprising different variants of NMB 1870.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

MULTIPLE VARIANTS OF MENINGOCOCCAL PROTEIN NMB1870

All documents cited herein are incorporated by reference in their entirety.

TECHNICAL FIELD

This invention is in the field of vaccination and, in particular, vaccination against disease caused by pathogenic bacteria in the genus *Neisseria*, such as *N.meningitidis* (meningococcus).

BACKGROUND ART

Neisseria meningitidis is a Gram-negative encapsulated bacterium which colonises the upper respiratory tract of approximately 10% of human population. Approximately once in every 10,000 colonised people (or once in 100,000 population) the bacterium enters the blood stream where it multiplies and causes sepsis. From the blood stream the bacterium can cross the blood-brain barrier and cause meningitis. Both diseases are devastating and can kill 5-15% of affected children and young adults within hours, despite the availability of effective antibiotics. Up to 25% of those who survive are left with permanent sequelae.

Prevention of disease has been partially accomplished by vaccination. Immunisation was made possible in 1969 when it was discovered that protection from disease correlates with the presence of serum antibodies able to induce complement-mediated killing of bacteria, and that purified capsular polysaccharide was able to induce these antibodies. Although polysaccharide and conjugate vaccines are available against serogroups A, C, W135 and Y, this approach cannot be applied to serogroup B because the capsular polysaccharide is a polymer of polysialic acid, which is a self antigen in humans. To develop a vaccine against serogroup B, surface-exposed proteins contained in outer membrane vesicles (OMVs) have been used. These vaccines elicit serum bactericidal antibody responses and protect against disease, but they fail to induce cross-strain protection [1].

The complete genome sequence of serogroup B *N.meningitidis* has been published [2] and has been subjected to analysis in order to identify vaccine antigens [3].The complete genome sequence of serogroup A *N.meningitidis* is also known [4], and the complete genome sequence of *Neisseria gonorrhoeae* strain FA1090 is available [5]. References 6 to 9 disclose proteins from *Neisseria meningitidis* and *Neisseria gonorrhoeae*, and approaches to expression of these proteins are disclosed in references 10 to 12.

It is an object of the invention to provide further and improved compositions for providing immunity against meningococcal disease and/or infection, particularly for serogroup B.

DISCLOSURE OF THE INVENTION

One of the ~2200 proteins disclosed in reference 2 is 'NMB1870'. The protein was originally disclosed as protein '741' from strain MC58 [SEQ IDs 2535 & 2536 in ref. 8; SEQ ID 1 herein], and has also been referred to as 'GNA1870' [following ref. 3] or as 'ORF2086' [13].

It has now been found that NMB1870 is an extremely effective antigen for eliciting anti-meningococcal antibody responses, and that it is expressed across all meningococcal serogroups.

NMB1870 has been found in all meningococcal strains tested to date. Forty-two different meningococcal NMB1870 sequences have been identified, and it has been found that these sequences group into three variants. Furthermore, it has been found that serum raised against a given variant is bactericidal within the same variant group, but is not active against strains which express one of the other two variants *i.e.* there is intra-variant cross-protection, but not inter-variant cross-protection. For maximum cross-strain efficacy, therefore, more than one variant should be used for immunising a patient.

The invention therefore provides a composition comprising at least two of the following antigens:

(a) a first protein, comprising an amino acid sequence having at least *a%* sequence identity to SEQ ID 24 and/or comprising an amino acid sequence consisting of a fragment of at least *x* contiguous amino acids from SEQ ID 24;

(b) a second protein, comprising an amino acid sequence having at least *b%* sequence identity to SEQ ID 33 and/or comprising an amino acid sequence consisting of a fragment of at least *y* contiguous amino acids from SEQ ID 33; and

(c) a third protein, comprising an amino acid sequence having at least *c%* sequence identity to SEQ ID 41 and/or comprising an amino acid sequence consisting of a fragment of at least *z* contiguous amino acids from SEQ ID 41.

The invention also provides the use of NMB1870 for providing immunity against multiple (*e.g.* 2, 3, 4, 5 or more) strains and/or serogroups of *N.meningitidis*.

Variability in and between (a), (b) and (c)

The value of *a* is at least 85 *e.g.* 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, or more. The value of *b* is at least 85 *e.g.* 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, or more. The value of *c* is at least 85 *e.g.* 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, or more. The values of *a, b* and *c* are not intrinsically related to each other.

The value of *x* is at least 7 *e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250). The value of *y* is at least 7 *e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250). The value of *z* is at least 7 *e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250). The values of *x, y* and *z* are not intrinsically related to each other.

It is preferred that any given amino acid sequence will not fall into more than one of categories (a), (b) and (c). Any given NMB1870 sequence will thus fall into only one of categories (a), (b) and (c). It is thus preferred that: protein (a) has less than *i%* sequence identity to protein (b); protein (a) has less than *j%* sequence identity to protein (c); and protein (b) has less than *k%* sequence identity to protein (c). The value of *i* is 60 or more (*e.g.* 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, *etc.*) and is at most *a*. The value of *j* is 60 or more (*e.g.* 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84,

85, 86, 87, 88, 89, 90, *etc.*) and is at most *b*. The value of *k* is 60 or more (*e.g.* 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, *etc.*) and is at most *c*. The values of *i, j* and *k* are not intrinsically related to each other.

In an example two-protein embodiment of the invention, therefore, protein (a) might have >85% sequence identity to SEQ ID 24, protein (b) might have >85% sequence identity to SEQ ID 33, but protein (a) and (b) have less than 75% sequence identity to each other. Proteins (a) and (b) are therefore each closely related to their 'prototype' sequences, but they are not so closely related to each other.

In an example three-protein embodiment of the invention, therefore, protein (a) might have >85% sequence identity to SEQ ID 24, protein (b) might have >85% sequence identity to SEQ ID 33, protein (c) might have >85% sequence identity to SEQ ID 41, but protein (a) and (b) have less than 75% sequence identity to each other, protein (a) and (c) have less than 75% sequence identity to each other, and protein (b) and (c) have less than 75% sequence identity to each other.

The mixture of two or more of (a), (b) and (c) can preferably elicit an antibody response which is bactericidal against at least one *N.meningitidis* strain from each of at least two of the following three groups of strains:

(a) MC58, gb185 (=M01-240185), m4030, m2197, m2937, iss1001, NZ394/98, 67/00, 93/114, bz198, m1390, nge28, lnp17592, 00-241341, f6124, 205900, m198/172, bz133, gb149 (=M01-240149), nm008, nm092, 30/00, 39/99, 72/00, 95330, bz169, bz83, cu385, h44/76, m1590, m2934, m2969, m3370, m4215, m4318, n44/89, 14847.

(b) 961-5945, 2996, 96217, 312294, 11327, a22, gb013 (=M01-240013), e32, m1090, m4287, 860800, 599, 95N477, 90-18311, c11, m986, m2671, 1000, m1096, m3279, bz232, dk353, m3697, ngh38, L93/4286.

(c) M1239, 16889, gb355 (=M01-240355), m3369, m3813, ngp165.

For example, the mixture can elicit a bactericidal response effective against each of serogroup B *N.meningitidis* strains MC58, 961-5945 and M1239.

The mixture of two or more of (a), (b) and (c) can preferably elicit an antibody response which is bactericidal against at least 50% of clinically-relevant meningococcal serogroup B strains (*e.g.* at least 60%, 70%, 80%, 90%, 95% or more).

The mixture of two or more of (a), (b) and (c) can preferably elicit an antibody response which is bactericidal against strains of serogroup B *N.meningitidis* and strains of at least one (*e.g.* 1, 2, 3, 4) of serogroups A, C, W135 and Y.

The mixture of two or more of (a), (b) and (c) can preferably elicit an antibody response which is bactericidal against strains of *N.gonococcus* and/or *N.cinerea*.

The mixture of two or more of (a), (b) and (c) can preferably elicit an antibody response which is bactericidal against strains from at least two of the three main branches of the dendrogram shown in

Figure 9 (*i.e.* the dendrogram obtained by analysing SEQ IDs 1 to 23 by the Kimura & Jukes-Cantor algorithm).

The mixture of two or more of (a), (b) and (c) can preferably elicit an antibody response which is bactericidal against *N.meningitidis* strains in at least 2 (*e.g.* 2, 3, 4, 5, 6, 7) of hypervirulent lineages ET-37, ET-5, cluster A4, lineage 3, subgroup I, subgroup III, and subgroup IV-1 [14, 15].

Compositions of the invention may additionally induce bactericidal antibody responses against one or more hyperinvasive lineages.

The mixture of two or more of (a), (b) and (c) can preferably elicit an antibody response which is bactericidal against *N.meningitidis* strains in at least at least 2 (*e.g.* 2, 3, 4, 5, 6, 7) of the following multilocus sequence types: ST1, ST4, ST5, ST8, ST11, ST32 and ST41 [16]. The mixture may also elicit an antibody response which is bactericidal against ST44 strains.

Bactericidal antibody responses are conveniently measured in mice and are a standard indicator of vaccine efficacy [*e.g.* see end-note 14 of reference 3]. The composition need not induce bactericidal antibodies against each and every MenB strain within the specified lineages or MLST; rather, for any given group of four of more strains of serogroup B meningococcus within a particular hypervirulent lineage or MLST, the antibodies induced by the composition are bactericidal against at least 50% (*e.g.* 60%, 70%, 80%, 90% or more) of the group. Preferred groups of strains will include strains isolated in at least four of the following countries: GB, AU, CA, NO, IT, US, NZ, NL, BR, and CU. The serum preferably has a bactericidal titre of at least 1024 (*e.g.* 2^{10}, 2^{11}, 2^{12}, 2^{13}, 2^{14}, 2^{15}, 2^{16}, 2^{17}, 2^{18} or higher, preferably at least 2^{14}) *i.e.* the serum is able to kill at least 50% of test bacteria of a particular strain when diluted 1/1024 *e.g.* as described in end-note 14 of reference 3.

Lipoproteins

NMB1870 is naturally a lipoprotein in *N.meningitidis*. It has also been found to be lipidated when expressed in *E.coli*.

It is preferred that one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) NMB1870 proteins included in compositions of the invention is a lipoprotein.

The invention provides a protein comprising an amino acid sequence having at least 50% (*e.g.* 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) sequence identity to one or more of SEQ IDs 24 to 45, and/or comprising an amino acid sequence consisting of a fragment of at least 7 (*e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250) contiguous amino acids from one or more of SEQ IDs 24 to 45 (preferably SEQ IDs 25 to 45), characterised in that the protein is a lipoprotein.

Preferably, the lipoprotein has a N-terminal cysteine residue, to which the lipid is covalently attached. To prepare the lipoprotein via bacterial expression generally requires a suitable N-terminal signal peptide to direct lipidation by diacylglyceryl transferase, followed by cleavage by lipoprotein-specific (type II) SPase. While the lipoprotein of the invention may have a N-terminal

cysteine (*e.g.* SEQ IDs 24 to 45), therefore, it will be a product of post-translational modification of a nascent protein which has the usual N-terminal methionine (*e.g.* SEQ IDs 1 to 22).

The lipoprotein may be associated with a lipid bilayer and may be solubilised with detergent.

Sequences

NMB1870 proteins useful for the invention comprise an amino acid sequence having at least 50% (*e.g.* 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) sequence identity to one or more of SEQ ID NO^{S} 1 to 23, and/or comprising an amino acid sequence consisting of a fragment of at least 7 (*e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250) contiguous amino acids from one or more of SEQ ID NO^{S} 1 to 23.

Preferred fragments include: (a) fragments which comprise an epitope, and preferably a bactericidal epitope; (b) fragments common to two or more of SEQ IDs 1 to 23; (c) SEQ IDs 1 to 23 with 1 or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120 or more, *etc.*) N-terminal residues deleted; (d) SEQ IDs 1 to 23 with 1 or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,17, 18, 19, 20, 21, 22, 23, 24, 25, *etc.*) C-terminal residues deleted; and (e) SEQ IDs 1 to 23 without their signal peptides (*e.g.* SEQ IDs 24 to 45). These preferred fragments are not mutually exclusive *e.g.* a fragment could fall into category (a) and (b), or category (c) and (d), *etc.*

Further NMB1870 proteins useful for the invention comprise an amino acid sequence having at least 50% (*e.g.* 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) sequence identity to one or more of SEQ ID NO^{S} 123 to 141, and/or comprising an amino acid sequence consisting of a fragment of at least 7 (*e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250) contiguous amino acids from one or more of SEQ ID NO^{S} 123 to 141.

Further NMB1870 proteins useful for the invention comprise an amino acid sequence having at least 50% (*e.g.* 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) sequence identity to one or more of SEQ ID NO^{S} 1 to 252 of reference 13, and/or comprising an amino acid sequence consisting of a fragment of at least 7 (*e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250) contiguous amino acids from one or more of SEQ ID NO^{S} 1 to 252 of reference 13. SEQ ID NO^{S} 300-302 of reference 13 provide consensus sequences, and SEQ ID NO^{S} 254-299 are fragments. Preferred fragments include: (a) fragments which comprise an epitope, and preferably a bactericidal epitope; (b) fragments common to two or more of SEQ IDs 123 to 141; (c) SEQ IDs 123 to 141 with 1 or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120 or more, *etc.*) N-terminal residues deleted; (d) SEQ IDs 123 to 141 with 1 or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,17, 18, 19, 20, 21, 22, 23, 24, 25, *etc.*) C-terminal residues deleted; and (e) SEQ IDs 123 to 141 without their signal

peptides. These preferred fragments are not mutually exclusive *e.g.* a fragment could fall into category (a) and (b), or category (c) and (d), *etc.*

Preferred amino acid sequences with <100% identity to SEQ ID NO^S 1 to 23 and 123 to 141 are allelic variants, homologs, orthologs, paralogs, mutants *etc.* thereof. It is preferred that one or more of the differences in allelic variants, homologs, orthologs, paralogs or mutants, compared to SEQ ID NO^S 1 to 23 and 123 to 141, involves a conservative amino acid replacement *i.e.* replacement of one amino acid with another which has a related side chain. Genetically-encoded amino acids are generally divided into four families: (1) acidic *i.e.* aspartate, glutamate; (2) basic *i.e.* lysine, arginine, histidine; (3) non-polar *i.e.* alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar *i.e.* glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In general, substitution of single amino acids within these families does not have a major effect on the biological activity.

A preferred subset of proteins does not include the amino acid sequence TRSKP (SEQ ID NO: 70) or TRSKPV (SEQ ID NO: 71) within 10 amino acids of the protein's N-terminus. Another preferred subset of proteins does not include the amino acid sequence PSEPPFG (SEQ ID NO: 72) within 10 amino acids of the protein's N-terminus.

Another preferred subset of proteins for use with the invention includes the amino acid sequence $(Gly)_n$, where n is 1, 2, 3, 4 or more *e.g.* SEQ ID NO: 73.

A characteristic of preferred proteins of the invention is the ability to induce bactericidal anti-meningococcal antibodies after administration to a host animal.

Proteins can be prepared by various means *e.g.* by chemical synthesis (at least in part), by digesting longer polypeptides using proteases, by translation from RNA, by purification from cell culture (*e.g.* from recombinant expression or from *N.meningitidis* culture). *etc.* Heterologous expression in an *E.coli* host is a preferred expression route (*e.g.* strains DH5α, BL21(DE_3), BLR, *etc.*).

Proteins of the invention may be attached or immobilised to a solid support.

Proteins of the invention may comprise a detectable label *e.g.* a radioactive label, a fluorescent label, or a biotin label. This is particularly useful in immunoassay techniques.

Proteins can take various forms (*e.g.* native, fusions, glycosylated, non-glycosylated, lipidated, disulfide bridges, *etc.*). Proteins are preferably meningococcal proteins.

Proteins are preferably prepared in substantially pure or substantially isolated form (*i.e.* substantially free from other Neisserial or host cell proteins) or substantially isolated form. In general, the proteins are provided in a non-naturally occurring environment *e.g.* they are separated from their naturally-occurring environment. In certain embodiments, the subject protein is present in a composition that is enriched for the protein as compared to a control. As such, purified protein is provided, whereby purified is meant that the protein is present in a composition that is substantially

free of other expressed proteins, where by substantially free is meant that less than 90%, usually less than 60% and more usually less than 50% of the composition is made up of other expressed proteins.

The term "protein" refers to amino acid polymers of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, proteins containing one or more analogs of an amino acid (including, for example, unnatural amino acids, *etc.*), as well as other modifications known in the art. Proteins can occur as single chains or associated chains.

The invention also provides proteins comprising an amino acid sequence having at least 50% (*e.g.* 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) sequence identity to one or more of SEQ ID NO^S 77, 79, 82, 83, 85, 87, 88, 89, 90, 91, 92, 93 & 94, and/or comprising an amino acid sequence consisting of a fragment of at least 7 (*e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250) contiguous amino acids from one or more of SEQ ID NO^S 77, 79, 82, 83, 85, 87, 88, 89, 90, 91, 92, 93 & 94.

Where the invention relates to a single NMB1870 protein, the invention does not encompass a protein comprising an amino acid sequence as disclosed in any of SEQ ID NO^S: 1 to 302 of reference 13. However, such proteins can optionally be used where the invention relates to NMB1870 mixtures

Hybrid and tandem proteins

As mentioned above, NMB1870 may be used in the form of a fusion protein, although the proteins may also be expressed other than as a fusion protein (*e.g.* without GST, MBP, his-tag or similar).

Fusion proteins can have a C-terminus and/or N-terminus fusion partner. Where a N-terminus fusion partner is used with SEQ IDs 1 to 23, the skilled person will realise that the start codon will (if included) be expressed as a valine, because GTG is translated as valine except when it is used as a start codon, in which case it is translated as N-formyl-methionine.

Suitable N-terminus fusion partners include leader peptides from other proteins (particularly other lipoproteins), which may be substituted for the natural NMB1870 leader peptides (*i.e.* the sequence prior to the N-terminus cysteine may be replaced with another leader peptide of interest). Examples are sequences comprising SEQ ID 46, and the *H.influenzae* P4 lipoprotein leader sequence [*e.g.* 17].

A preferred type of fusion protein is disclosed in references 10, 11 & 12 in which two or more (*e.g.* 3, 4, 5, 6 or more) Neisserial proteins are joined such that they are translated as a single polypeptide chain. In general, such hybrid proteins can be represented by the formula:

$$NH_2\text{-A-[-X-L-]}_n\text{-B-COOH}$$

wherein X is an amino acid sequence comprising a Neisserial sequence, L is an optional linker amino acid sequence, A is an optional N-terminal amino acid sequence, B is an optional C-terminal amino

acid sequence, and *n* is an integer greater than 1. The value of *n* is between 2 and *x*, and the value of *x* is typically 3, 4, 5, 6, 7, 8, 9 or 10. Preferably *n* is 2, 3 or 4; it is more preferably 2 or 3; most preferably, $n = 2$.

According to the present invention, at least one of the -X- moieties is a NMB1870 sequence as defined above. In some hybrid proteins, referred to as 'tandem' proteins, at least one of the -X- moieties has sequence identity to at least one of the other X moieties *e.g.* X_1 is SEQ ID NO: 24 and X_2 is a SEQ ID NO: 25. Proteins in which two or three of the three NMB1870 variants are joined as a tandem protein are preferred.

For X moieties other than X_1, it is preferred that the native leader peptide should be omitted, particularly where X_1 is not a NMB1870 sequence. In one embodiment, the leader peptides will be deleted except for that of the -X- moiety located at the N-terminus of the hybrid protein *i.e.* the leader peptide of X_1 will be retained, but the leader peptides of X_2 ... X_n will be omitted. This is equivalent to deleting all leader peptides and using the leader peptide of X_1 as moiety -A-.

Preferred NMB1870 sequences for use as -X- moieties are truncated up to and including the poly-glycine sequence found near the mature N-terminus *e.g.* the NMB1870 sequence will begin VAA... (or IAA... for strain m3813). Such NMB1870 sequences include SEQ ID NO^S: 80, 81 & 84.

For each *n* instances of [-X-L-], linker amino acid sequence -L- may be present or absent. For instance, when n=2 the hybrid may be NH_2-X_1-L_1-X_2-L_2-COOH, NH_2-X_1-X_2-COOH, NH_2-X_1-L_1-X_2-COOH, NH_2-X_1-X_2-L_2-COOH, *etc.* Linker amino acid sequence(s) -L- will typically be short (*e.g.* 20 or fewer amino acids *i.e.* 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include short peptide sequences which facilitate cloning, poly-glycine linkers (*i.e.* Gly_n where $n = 2$, 3, 4, 5, 6, 7, 8, 9, 10 or more), and histidine tags (*i.e.* His_n where $n = 3$, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable linker amino acid sequences will be apparent to those skilled in the art. A useful linker is GSGGGG (SEQ ID NO: 144), with the Gly-Ser dipeptide being formed from a *Bam*HI restriction site, thus aiding cloning and manipulation, and the Gly_4 tetrapeptide (SEQ ID NO: 73) is another typical poly-glycine linker. Another useful linker is SEQ ID NO: 78.

-A- is an optional N-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include leader sequences to direct protein trafficking, or short peptide sequences which facilitate cloning or purification (*e.g.* histidine tags *i.e.* His_n where $n = 3$, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable N-terminal amino acid sequences will be apparent to those skilled in the art. If X_1 lacks its own N-terminus methionine, -A- may provide such a methionine residue in the translated protein (*e.g.* -A- is a single Met residue). A useful -A- moiety for expressing NMB1870 is SEQ ID NO: 86. In mature lipoproteins, -A- preferably provides a N-terminus cysteine (*e.g.* -A- is a single Cys residue).

-B- is an optional C-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include sequences to direct protein

trafficking, short peptide sequences which facilitate cloning or purification (*e.g.* comprising histidine tags *i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more), or sequences which enhance protein stability. Other suitable C-terminal amino acid sequences will be apparent to those skilled in the art.

In preferred hybrid proteins of the invention, one of the X moieties is a 'protein 936' sequence. For example, where n = 2, A = Met, X_1 is a 936 sequence (*e.g.* SEQ ID NO: 76, which is the processed MC58 protein), L_1 = a poly-glycine linker (*e.g.* SEQ ID NO: 144), X_2 = a NMB1870 sequence in which the N-terminus has been deleted up to and including its own poly-glycine sequence, and L_2 and B may be omitted. An example of such a hybrid protein is SEQ ID NO: 77, in which truncated NMB1870 from strain m1239 is downstream of the processed 936 from strain MC58. Further examples of hybrid proteins of 936 (2996 strain) and truncated NMB1870 (strain 2996 or M1239) are SEQ ID NO^S: 91, 92, 93 & 94.

Preferred tandem proteins where n=3 may have all three NMB1870 variants in any order:

X_1	1	1	2	2	3	3
X_2	2	3	1	3	1	2
X_3	3	2	3	1	2	1

Preferred tandem proteins where n=2 may have two different NMB1870 variants:

X_1	1	1	2	2	3	3
X_2	2	3	1	3	1	2

Examples of tandem proteins where n=2 (two different NMB1870 variants) are SEQ ID NO^S: 79, 82, 83, 85, 87, 88, 89 & 90, which use strains MC58 (variant 1), 2996 (variant 2) and M1239 (variant 3).

An example of a tandem protein where n=3 is given as SEQ ID NO: 142.

NadA

NadA protein is disclosed in references 191 and 192. These references disclose three distinct alleles of NadA, although some minor variations were found *e.g.* serogroup C strain ISS1024 has a variant of allele 2 with a single heptad repeat deletion, serogroup C strains ISS759 and 973-1720 both contain a variant of allele 3 with a single amino acid mutation in the leader peptide, and serogroup B strain 95330 contains a recombination of alleles 1 and 2.

In sequencing NadA from Haji strains of meningococcus, SEQ ID NO: 143 was identified. This protein is a recombinant of known alleles 2 and 3.

The invention provides a protein comprising an amino acid sequence having at least 50% (*e.g.* 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or more *e.g.* 100%) sequence identity to SEQ ID NO: 143, and/or comprising an amino acid sequence consisting of a fragment of at least 7 (*e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250) contiguous amino acids from SEQ ID NO: 143.

Preferred fragments include: (a) fragments which comprise an epitope, and preferably a bactericidal epitope; (b) fragments common to SEQ ID NO: 143 and at least one of the NadA sequences disclosed in references 191 and 192; (c) SEQ ID NO: 143 with 1 or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120 or more, etc.) N-terminal residues deleted; (d) SEQ ID NO: 143 with 1 or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,17, 18, 19, 20, 21, 22, 23, 24, 25, etc.) C-terminal residues deleted; and (e) SEQ ID NO: 143 without its signal peptide. These preferred fragments are not mutually exclusive e.g. a fragment could fall into category (a) and (b), or category (c) and (d), etc.

Preferred amino acid sequences with <100% identity to SEQ ID NO: 143 are allelic variants, homologs, orthologs, paralogs, mutants etc. thereof. It is preferred that one or more of the differences in allelic variants, homologs, orthologs, paralogs or mutants, compared to SEQ ID NO: 143, involves a conservative amino acid replacement.

Nucleic acids

The invention provides nucleic acid encoding a protein of the invention as defined above. The invention also provides nucleic acid comprising: (a) a fragment of at least *n* consecutive nucleotides from said nucleic acid, wherein *n* is 10 or more (*e.g.* 12, 14, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500 or more); and/or (b) a sequence having at least 50% (*e.g.* 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or more) sequence identity to said nucleic acid.

Furthermore, the invention provides nucleic acid which can hybridise to nucleic acid encoding a protein of the invention, preferably under "high stringency" conditions (*e.g.* 65°C in a 0.1xSSC, 0.5% SDS solution).

Nucleic acids of the invention can be used in hybridisation reactions (*e.g.* Northern or Southern blots, or in nucleic acid microarrays or 'gene chips') and amplification reactions (*e.g.* PCR, SDA, SSSR, LCR, TMA, NASBA, *etc.*) and other nucleic acid techniques.

Nucleic acids of the invention can be prepared in many ways *e.g.* by chemical synthesis in whole or part, by digesting longer polynucleotides using nucleases (*e.g.* restriction enzymes), from genomic or cDNA libraries, from the bacterium itself, *etc.*

Nucleic acids of the invention can take various forms *e.g.* single-stranded, double-stranded, vectors, primers, probes, labelled, unlabelled, *etc.*

Nucleic acids of the invention are preferably in isolated or substantially isolated form.

The invention includes nucleic acid comprising sequences complementary to those described above *e.g.* for antisense or probing, or for use as primers.

The term "nucleic acid" includes DNA and RNA, and also their analogues, such as those containing modified backbones, and also peptide nucleic acids (PNA) *etc.*

Nucleic acid according to the invention may be labelled *e.g.* with a radioactive or fluorescent label. This is particularly useful where the nucleic acid is to be used in nucleic acid detection techniques

e.g. where the nucleic acid is a primer or as a probe for use in techniques such as PCR, LCR, TMA, NASBA, *etc.*

The invention also provides vectors comprising nucleotide sequences of the invention (*e.g.* cloning or expression vectors, such as those suitable for nucleic acid immunisation) and host cells transformed with such vectors.

Further antigenic components

Compositions of the invention include a small number (*e.g.* fewer than *t* antigens, where *t* is 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4 or 3) of purified serogroup B antigens. It is particularly preferred that the composition should not include complex or undefined mixtures of antigens *e.g.* it is preferred not to include outer membrane vesicles in the composition. The antigens are preferably expressed recombinantly in a heterologous host and then purified.

The composition of the invention includes at least two different NMB1870 proteins. It may also include another neisserial antigen, as a vaccine which targets more than one antigen per bacterium decreases the possibility of selecting escape mutants. Neisserial antigens for inclusion in the compositions include proteins comprising:

(a) the 446 even SEQ IDs (*i.e.* 2, 4, 6, ... , 890, 892) disclosed in reference 6.
(b) the 45 even SEQ IDs (i.e. 2, 4, 6, ... , 88, 90) disclosed in reference 7;
(c) the 1674 even SEQ IDs 2-3020, even SEQ IDs 3040-3114, and all SEQ IDs 3115-3241, disclosed in reference 8;
(d) the 2160 amino acid sequences NMB0001 to NMB2160 from reference 2;
(e) an amino acid sequence disclosed in reference 10, 11 or 12;
(f) a variant, homolog, ortholog, paralog, mutant *etc.* of (a) to (e); or
(g) an outer membrane vesicle prepared from *N.meningitidis* [*e.g.* see ref. 139].

In addition to Neisserial antigens, the composition may include antigens for immunising against other diseases or infections. For example, the composition may include one or more of the following further antigens:

– antigens from *Helicobacter pylori* such as CagA [18 to 21], VacA [22, 23], NAP [24, 25, 26], HopX [*e.g.* 27], HopY [*e.g.* 27] and/or urease.
– a saccharide antigen from *N.meningitidis* serogroup A, C, W135 and/or Y, such as the oligosaccharide disclosed in ref. 28 from serogroup C [see also ref. 29] or the oligosaccharides of ref. 30.
– a saccharide antigen from *Streptococcus pneumoniae* [*e.g.* 31, 32, 33].
– an antigen from hepatitis A virus, such as inactivated virus [*e.g.* 34, 35].
– an antigen from hepatitis B virus, such as the surface and/or core antigens [*e.g.* 35, 36].
– a diphtheria antigen, such as a diphtheria toxoid [*e.g.* chapter 3 of ref. 37] *e.g.* the CRM_{197} mutant [*e.g.* 38].
– a tetanus antigen, such as a tetanus toxoid [*e.g.* chapter 4 of ref. 37].

- an antigen from *Bordetella pertussis*, such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 [*e.g.* refs. 39 & 40].
- a saccharide antigen from *Haemophilus influenzae* B [*e.g.* 29].
- an antigen from hepatitis C virus [*e.g.* 41].
- an antigen from *N.gonorrhoeae* [*e.g.* 6, 7, 8, 42].
- an antigen from *Chlamydia pneumoniae* [*e.g.* refs. 43 to 49].
- an antigen from *Chlamydia trachomatis* [*e.g.* 50].
- an antigen from *Porphyromonas gingivalis* [*e.g.* 51].
- polio antigen(s) [*e.g.* 52, 53] such as IPV.
- rabies antigen(s) [*e.g.* 54] such as lyophilised inactivated virus [*e.g.* 55, RabAvert™].
- measles, mumps and/or rubella antigens [*e.g.* chapters 9, 10 & 11 of ref. 37].
- influenza antigen(s) [*e.g.* chapter 19 of ref. 37], such as the haemagglutinin and/or neuraminidase surface proteins.
- an antigen from *Moraxella catarrhalis* [*e.g.* 56].
- an protein antigen from *Streptococcus agalactiae* (group B streptococcus) [*e.g.* 57, 58].
- a saccharide antigen from *Streptococcus agalactiae* (group B streptococcus).
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [*e.g.* 58, 59, 60].
- an antigen from *Staphylococcus aureus* [*e.g.* 61].
- an antigen from *Bacillus anthracis* [*e.g.* 62, 63, 64].
- an antigen from a virus in the flaviviridae family (genus flavivirus), such as from yellow fever virus, Japanese encephalitis virus, four serotypes of Dengue viruses, tick-borne encephalitis virus, West Nile virus.
- a pestivirus antigen, such as from classical porcine fever virus, bovine viral diarrhoea virus, and/or border disease virus.
- a parvovirus antigen *e.g.* from parvovirus B19.
- a prion protein (*e.g.* the CJD prion protein)
- an amyloid protein, such as a beta peptide [65]
- a cancer antigen, such as those listed in Table 1 of ref. 66 or in tables 3 & 4 of ref. 67.

The composition may comprise one or more of these further antigens.

Toxic protein antigens may be detoxified where necessary (*e.g.* detoxification of pertussis toxin by chemical and/or genetic means [40]).

Where a diphtheria antigen is included in the composition it is preferred also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is preferred also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is preferred also to include diphtheria and tetanus antigens. DTP combinations are thus preferred.

Saccharide antigens are preferably in the form of conjugates. Carrier proteins for the conjugates include the *N.meningitidis* outer membrane protein [68], synthetic peptides [69,70], heat shock

proteins [71,72], pertussis proteins [73,74], protein D from *H.influenzae* [75], cytokines [76], lymphokines [76], streptococcal proteins, hormones [76], growth factors [76], toxin A or B from *C.difficile* [77], iron-uptake proteins [78], *etc.* A preferred carrier protein is the CRM197 diphtheria toxoid [79].

Antigens in the composition will typically be present at a concentration of at least 1μg/ml each. In general, the concentration of any given antigen will be sufficient to elicit an immune response against that antigen.

Immunogenic compositions of the invention may be used therapeutically (*i.e.* to treat an existing infection) or prophylactically (*i.e.* to prevent future infection).

As an alternative to using proteins antigens in the immunogenic compositions of the invention, nucleic acid (preferably DNA *e.g.* in the form of a plasmid) encoding the antigen may be used.

Particularly preferred compositions of the invention include one, two or three of: (a) saccharide antigens from meningococcus serogroups Y, W135, C and (optionally) A; (b) a saccharide antigen from *Haemophilus influenzae* type B; and/or (c) an antigen from *Streptococcus pneumoniae*.

Meningococcus serogroups Y, W135, C and (optionally) A

Polysaccharide vaccines against serogroups A, C, W135 & Y have been known for many years. These vaccines (MENCEVAX ACWY™ and MENOMUNE™) are based on the organisms' capsular polysaccharides and, although they are effective in adolescents and adults, they give a poor immune response and short duration of protection, and they cannot be used in infants.

In contrast to the unconjugated polysaccharide antigens in these vaccines, the recently-approved serogroup C vaccines (Menjugate™ [80,28], Meningitec™ and NeisVac-C™) include conjugated saccharides. Menjugate™ and Meningitec™ have oligosaccharide antigens conjugated to a CRM_{197} carrier, whereas NeisVac-C™ uses the complete polysaccharide (de-O-acetylated) conjugated to a tetanus toxoid carrier. The proposed MenActra™ vaccine contains conjugated capsular saccharide antigens from each of serogroups Y, W135, C and A.

Compositions of the present invention preferably include capsular saccharide antigens from one or more of meningococcus serogroups Y, W135, C and (optionally) A, wherein the antigens are conjugated to carrier protein(s) and/or are oligosaccharides. For example, the composition may include a capsular saccharide antigen from: serogroup C; serogroups A and C; serogroups A, C and W135; serogroups A, C and Y; serogroups C, W135 and Y; or from all four of serogroups A, C, W135 and Y.

A typical quantity of each meningococcal saccharide antigen per dose is between 1μg and 20μg *e.g.* about 1μg, about 2.5μg, about 4μg, about 5μg, or about 10μg (expressed as saccharide).

Where a mixture comprises capsular saccharides from both serogroups A and C, the ratio (w/w) of MenA saccharide:MenC saccharide may be greater than 1 (*e.g.* 2:1, 3:1, 4:1, 5:1, 10:1 or higher). Where a mixture comprises capsular saccharides from serogroup Y and one or both of serogroups C and W135, the ratio (w/w) of MenY saccharide:MenW135 saccharide may be greater than 1 (*e.g.*

2:1, 3:1, 4:1, 5:1, 10:1 or higher) and/or that the ratio (w/w) of MenY saccharide:MenC saccharide may be less than 1 (*e.g.* 1:2, 1:3, 1:4, 1:5, or lower). Preferred ratios (w/w) for saccharides from serogroups A:C:W135:Y are: 1:1:1:1; 1:1:1:2; 2:1:1:1; 4:2:1:1; 8:4:2:1; 4:2:1:2; 8:4:1:2; 4:2:2:1; 2:2:1:1; 4:4:2:1; 2:2:1:2; 4:4:1:2; and 2:2:2:1. Preferred ratios (w/w) for saccharides from serogroups C:W135:Y are: 1:1:1; 1:1:2; 1:1:1; 2:1:1; 4:2:1; 2:1:2; 4:1:2; 2:2:1; and 2:1:1. Using a substantially equal mass of each saccharide is preferred.

Capsular saccharides will generally be used in the form of oligosaccharides. These are conveniently formed by fragmentation of purified capsular polysaccharide (*e.g.* by hydrolysis), which will usually be followed by purification of the fragments of the desired size.

Fragmentation of polysaccharides is preferably performed to give a final average degree of polymerisation (DP) in the oligosaccharide of less than 30 (*e.g.* between 10 and 20, preferably around 10 for serogroup A; between 15 and 25 for serogroups W135 and Y, preferably around 15-20; between 12 and 22 for serogroup C; *etc.*). DP can conveniently be measured by ion exchange chromatography or by colorimetric assays [81].

If hydrolysis is performed, the hydrolysate will generally be sized in order to remove short-length oligosaccharides [29]. This can be achieved in various ways, such as ultrafiltration followed by ion-exchange chromatography. Oligosaccharides with a degree of polymerisation of less than or equal to about 6 are preferably removed for serogroup A, and those less than around 4 are preferably removed for serogroups W135 and Y.

Preferred MenC saccharide antigens are disclosed in reference 80, as used in Menjugate™.

The saccharide antigen may be chemically modified. This is particularly useful for reducing hydrolysis for serogroup A [82; see below]. De-O-acetylation of meningococcal saccharides can be performed. For oligosaccharides, modification may take place before or after depolymerisation.

Where a composition of the invention includes a MenA saccharide antigen, the antigen is preferably a modified saccharide in which one or more of the hydroxyl groups on the native saccharide has/have been replaced by a blocking group [82]. This modification improves resistance to hydrolysis.

The number of monosaccharide units having blocking groups can vary. For example, all or substantially all the monosaccharide units may have blocking groups. Alternatively, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the monosaccharide units may have blocking groups. At least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 monosaccharide units may have blocking groups.

Likewise, the number of blocking groups on a monosaccharide unit may vary. For example, the number of blocking groups on a monosaccharide unit may be 1 or 2. The blocking group will generally be at the 4 position and/or 3-position of the monosaccharide units.

The terminal monosaccharide unit may or may not have a blocking group instead of its native hydroxyl. It is preferred to retain a free anomeric hydroxyl group on a terminal monosaccharide unit

in order to provide a handle for further reactions (*e.g.* conjugation). Anomeric hydroxyl groups can be converted to amino groups ($-NH_2$ or –NH-E, where E is a nitrogen protecting group) by reductive amination (using, for example, $NaBH_3CN/NH_4Cl$), and can then be regenerated after other hydroxyl groups have been converted to blocking groups.

Blocking groups to replace hydroxyl groups may be directly accessible via a derivatizing reaction of the hydroxyl group *i.e.* by replacing the hydrogen atom of the hydroxyl group with another group. Suitable derivatives of hydroxyl groups which act as blocking groups are, for example, carbamates, sulfonates, carbonates, esters, ethers (*e.g.* silyl ethers or alkyl ethers) and acetals. Some specific examples of such blocking groups are allyl, Aloc, benzyl, BOM, t-butyl, trityl, TBS, TBDPS, TES, TMS, TIPS, PMB, MEM, MOM, MTM, THP, *etc.* Other blocking groups that are not directly accessible and which completely replace the hydroxyl group include C_{1-12} alkyl, C_{3-12} alkyl, C_{5-12} aryl, C_{5-12} aryl-C_{1-6} alkyl, NR^1R^2 (R^1 and R^2 are defined in the following paragraph), H, F, Cl, Br, CO_2H, $CO_2(C_{1-6}$ alkyl), CN, CF_3, CCl_3, *etc.* Preferred blocking groups are electron-withdrawing groups.

Preferred blocking groups are of the formula: –O–X–Y or $-OR^3$ wherein: X is C(O), S(O) or SO_2; Y is C_{1-12} alkyl, C_{1-12} alkoxy, C_{3-12} cycloalkyl, C_{5-12} aryl or C_{5-12} aryl-C_{1-6} alkyl, each of which may optionally be substituted with 1, 2 or 3 groups independently selected from F, Cl, Br, CO_2H, $CO_2(C_{1-6}$ alkyl), CN, CF_3 or CCl_3; or Y is NR^1R^2; R^1 and R^2 are independently selected from H, C_{1-12} alkyl, C_{3-12} cycloalkyl, C_{5-12} aryl, C_{5-12} aryl-C_{1-6} alkyl; or R^1 and R^2 may be joined to form a C_{3-12} saturated heterocyclic group; R^3 is C_{1-12} alkyl or C_{3-12} cycloalkyl, each of which may optionally be substituted with 1, 2 or 3 groups independently selected from F, Cl, Br, $CO_2(C_{1-6}$ alkyl), CN, CF_3 or CCl_3; or R^3 is C_{5-12} aryl or C_{5-12} aryl-C_{1-6} alkyl, each of which may optionally be substituted with 1, 2, 3, 4 or 5 groups selected from F, Cl, Br, CO_2H, $CO_2(C_{1-6}$ alkyl), CN, CF_3 or CCl_3. When R^3 is C_{1-12} alkyl or C_{3-12} cycloalkyl, it is typically substituted with 1, 2 or 3 groups as defined above. When R^1 and R^2 are joined to form a C_{3-12} saturated heterocyclic group, it is meant that R^1 and R^2 together with the nitrogen atom form a saturated heterocyclic group containing any number of carbon atoms between 3 and 12 (*e.g.* C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}). The heterocyclic group may contain 1 or 2 heteroatoms (such as N, O or S) other than the nitrogen atom. Examples of C_{3-12} saturated heterocyclic groups are pyrrolidinyl, piperidinyl, morpholinyl, piperazinyl, imidazolidinyl, azetidinyl and aziridinyl.

Blocking groups -O-X-Y and $-OR^3$ can be prepared from -OH groups by standard derivatizing procedures, such as reaction of the hydroxyl group with an acyl halide, alkyl halide, sulfonyl halide, *etc.* Hence, the oxygen atom in -O-X-Y is preferably the oxygen atom of the hydroxyl group, while the -X-Y group in -O-X-Y preferably replaces the hydrogen atom of the hydroxyl group.

Alternatively, the blocking groups may be accessible via a substitution reaction, such as a Mitsonobu-type substitution. These and other methods of preparing blocking groups from hydroxyl groups are well known.

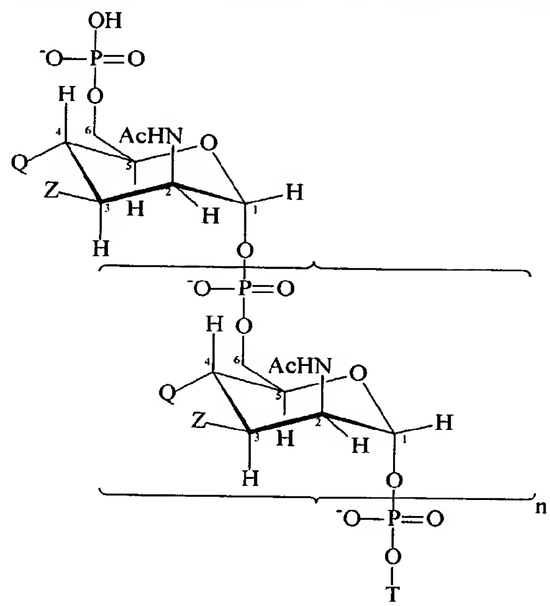
More preferably, the blocking group is $-OC(O)CF_3$ [83], or a carbamate group $-OC(O)NR^1R^2$, where R^1 and R^2 are independently selected from C_{1-6} alkyl. More preferably, R^1 and R^2 are both methyl *i.e.*

the blocking group is $-OC(O)NMe_2$. Carbamate blocking groups have a stabilizing effect on the glycosidic bond and may be prepared under mild conditions.

Preferred modified MenA saccharides contain *n* monosaccharide units, where at least *h*% of the monosaccharide units do not have –OH groups at both of positions 3 and 4. The value of *h* is 24 or more (*e.g.* 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 98, 99 or 100) and is preferably 50 or more. The absent –OH groups are preferably blocking groups as defined above.

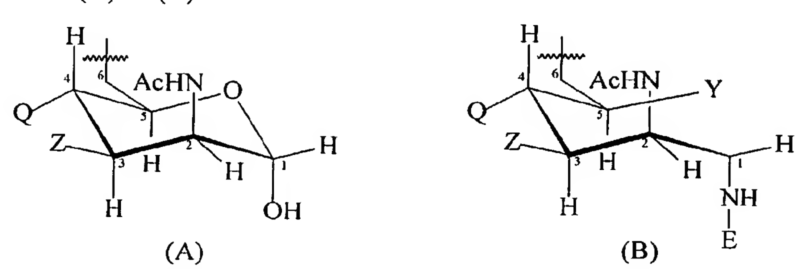
Other preferred modified MenA saccharides comprise monosaccharide units, wherein at least *s* of the monosaccharide units do not have–OH at the 3 position and do not have –OH at the 4 position. The value of *s* is at least 1 (*e.g.* 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90). The absent –OH groups are preferably blocking groups as defined above.

Suitable modified MenA saccharides for use with the invention have the formula:



, wherein

n is an integer from 1 to 100 (preferably an integer from 15 to 25);
T is of the formula (A) or (B):



each Z group is independently selected from OH or a blocking group as defined above; and
each Q group is independently selected from OH or a blocking group as defined above;
Y is selected from OH or a blocking group as defined above;
E is H or a nitrogen protecting group;

and wherein more than about 7% (*e.g.* 8%, 9%, 10% or more) of the Q groups are blocking groups.

Each of the *n+2* Z groups may be the same or different from each other. Likewise, each of the *n+2* Q groups may be the same or different from each other. All the Z groups may be OH. Alternatively, at least 10%, 20, 30%, 40%, 50% or 60% of the Z groups may be OAc. Preferably, about 70% of the Z groups are OAc, with the remainder of the Z groups being OH or blocking groups as defined above. At least about 7% of Q groups are blocking groups. Preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or even 100% of the Q groups are blocking groups.

Meningococcal capsular polysaccharides are typically prepared by a process comprising the steps of polysaccharide precipitation (*e.g.* using a cationic detergent), ethanol fractionation, cold phenol extraction (to remove protein) and ultracentrifugation (to remove LPS) [*e.g.* ref. 84]. A more preferred process [30], however, involves polysaccharide precipitation followed by solubilisation of the precipitated polysaccharide using a lower alcohol. Precipitation can be achieved using a cationic detergent such as tetrabutylammonium and cetyltrimethylammonium salts (*e.g.* the bromide salts), or hexadimethrine bromide and myristyltrimethylammonium salts. Cetyltrimethylammonium bromide ('CTAB') is particularly preferred [85]. Solubilisation of the precipitated material can be achieved using a lower alcohol such as methanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol, 2-methyl-propan-1-ol, 2-methyl-propan-2-ol, diols, *etc.*, but ethanol is particularly suitable for solubilising CTAB-polysaccharide complexes. Ethanol is preferably added to the precipitated polysaccharide to give a final concentration (based on total content of ethanol and water) of between 50% and 95%.

After re-solubilisation, the polysaccharide may be further treated to remove contaminants. This is particularly important in situations where even minor contamination is not acceptable (*e.g.* for human vaccine production). This will typically involve one or more steps of filtration *e.g.* depth filtration, filtration through activated carbon may be used, size filtration and/or ultrafiltration. Once filtered to remove contaminants, the polysaccharide may be precipitated for further treatment and/or processing. This can be conveniently achieved by exchanging cations (*e.g.* by the addition of calcium or sodium salts).

As an alternative to purification, capsular saccharides of the present invention may be obtained by total or partial synthesis *e.g.* Hib synthesis is disclosed in ref. 86, and MenA synthesis in ref. 87.

Compositions of the invention comprise capsular saccharides from at least two serogroups of *N.meningitidis*. The saccharides are preferably prepared separately (including any fragmentation, conjugation, modification, *etc.*) and then admixed to give a composition of the invention.

Where the composition comprises capsular saccharide from serogroup A, however, it is preferred that the serogroup A saccharide is not combined with the other saccharide(s) until shortly before use, in order to minimise the potential for hydrolysis. This can conveniently be achieved by having the serogroup A component (typically together with appropriate excipients) in lyophilised form and the other serogroup component(s) in liquid form (also with appropriate excipients), with the liquid components being used to reconstitute the lyophilised MenA component when ready for use. Where

an aluminium salt adjuvant is used, it is preferred to include the adjuvant in the vial containing the with the liquid vaccine, and to lyophilise the MenA component without adjuvant.

A composition of the invention may thus be prepared from a kit comprising: (a) capsular saccharide from *N.meningitidis* serogroup A, in lyophilised form; and (b) the further antigens from the composition, in liquid form. The invention also provides a method for preparing a composition of the invention, comprising mixing a lyophilised capsular saccharide from *N.meningitidis* serogroup A with the further antigens, wherein said further antigens are in liquid form.

The invention also provides a kit comprising: (a) a first container containing capsular saccharides from two or more of *N.meningitidis* serogroups C, W135 and Y, all in lyophilised form; and (b) a second container containing in liquid form (i) a composition which, after administration to a subject, is able to induce an antibody response in that subject, wherein the antibody response is bactericidal against two or more (*e.g.* 2 or 3) of hypervirulent lineages A4, ET-5 and lineage 3 of *N.meningitidis* serogroup B, (ii) capsular saccharides from none or one of *N.meningitidis* serogroups C, W135 and Y, and optionally (iii) further antigens (see below) that do not include meningococcal capsular saccharides, wherein, reconstitution of the contents of container (a) by the contents of container (b) provides a composition of the invention.

Within each dose, the amount of an individual saccharide antigen will generally be between 1-50 μg (measured as mass of saccharide), with about 2.5μg, 5μg or 10 μg of each being preferred. With A:C:W135:Y weight ratios of 1:1:1:1; 1:1:1:2; 2:1:1:1; 4:2:1:1; 8:4:2:1; 4:2:1:2; 8:4:1:2; 4:2:2:1; 2:2:1:1; 4:4:2:1; 2:2:1:2; 4:4:1:2; and 2:2:2:1, therefore, the amount represented by the number 1 is preferably about 2.5μg, 5μg or 10 μg. For a 1:1:1:1 ratio A:C:W:Y composition and a 10μg per saccharide, therefore, 40 μg saccharide is administered per dose. Preferred compositions have about the following μg saccharide per dose:

A	10	0	0	0	10	5	2.5
C	10	10	5	2.5	5	5	2.5
W135	10	10	5	2.5	5	5	2.5
Y	10	10	5	2.5	5	5	2.5

Preferred compositions of the invention comprise less than 50 μg meningococcal saccharide per dose. Other preferred compositions comprise ≤40 μg meningococcal saccharide per dose. Other preferred compositions comprise ≤30 μg meningococcal saccharide per dose. Other preferred compositions comprise ≤25 μg meningococcal saccharide per dose. Other preferred compositions comprise ≤20 μg meningococcal saccharide per dose. Other preferred compositions comprise ≤10 μg meningococcal saccharide per dose but, ideally, compositions of the invention comprise at least 10 μg meningococcal saccharide per dose.

The Menjugate™ and NeisVac™ MenC conjugates use a hydroxide adjuvant, whereas Meningitec™ uses a phosphate. It is possible in compositions of the invention to adsorb some antigens to an aluminium hydroxide but to have other antigens in association with an aluminium phosphate. For tetravalent serogroup combinations, for example, the following permutations are available:

Serogroup	**Aluminium salt** (H = a hydroxide; P = a phosphate)															
A	P	H	P	H	H	H	P	P	P	H	H	H	P	P	P	H
C	P	H	H	P	H	H	P	H	H	P	P	H	P	H	P	P
W135	P	H	H	H	P	H	H	P	H	H	P	P	P	P	H	P
Y	P	H	H	H	H	P	H	H	P	P	H	P	H	P	P	P

For trivalent *N.meningitidis* serogroup combinations, the following permutations are available:

Serogroup	**Aluminium salt** (H = a hydroxide; P = a phosphate)							
C	P	H	H	H	P	P	P	H
W135	P	H	H	P	H	P	H	P
Y	P	H	P	H	H	H	P	P

<u>Haemophilus influenzae type B</u>

Where the composition includes a *H.influenzae* type B antigen, it will typically be a Hib capsular saccharide antigen. Saccharide antigens from *H.influenzae* b are well known.

Advantageously, the Hib saccharide is covalently conjugated to a carrier protein, in order to enhance its immunogenicity, especially in children. The preparation of polysaccharide conjugates in general, and of the Hib capsular polysaccharide in particular, is well documented [*e.g.* references 88 to 96 *etc.*]. The invention may use any suitable Hib conjugate. Suitable carrier proteins are described below, and preferred carriers for Hib saccharides are CRM_{197} ('HbOC'), tetanus toxoid ('PRP-T') and the outer membrane complex of *N.meningitidis* ('PRP-OMP').

The saccharide moiety of the conjugate may be a polysaccharide (*e.g.* full-length polyribosylribitol phosphate (PRP)), but it is preferred to hydrolyse polysaccharides to form oligosaccharides (*e.g.* MW from ~1 to ~5 kDa).

A preferred conjugate comprises a Hib oligosaccharide covalently linked to CRM_{197} via an adipic acid linker [97, 98]. Tetanus toxoid is also a preferred carrier.

Compositions of the invention may comprise more than one Hib antigen.

Where a composition includes a Hib saccharide antigen, it is preferred that it does not also include an aluminium hydroxide adjuvant. If the composition includes an aluminium phosphate adjuvant then the Hib antigen may be adsorbed to the adjuvant [99] or it may be non-adsorbed [100].

Hib antigens may be lyophilised *e.g.* together with meningococcal antigens.

<u>Streptococcus pneumoniae</u>

Where the composition includes a *S.pneumoniae* antigen, it will typically be a capsular saccharide antigen which is preferably conjugated to a carrier protein [*e.g.* refs. 31-33]. It is preferred to include saccharides from more than one serotype of *S.pneumoniae*. For example, mixtures of polysaccharides from 23 different serotype are widely used, as are conjugate vaccines with polysaccharides from between 5 and 11 different serotypes [101]. For example, PrevNar™ [102] contains antigens from seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) with each saccharide individually conjugated to CRM_{197} by reductive amination, with 2μg of each saccharide per 0.5ml dose (4μg of serotype 6B),

and with conjugates adsorbed on an aluminium phosphate adjuvant. Compositions of the invention preferably include at least serotypes 6B, 14, 19F and 23F. Conjugates may be adsorbed onto an aluminium phosphate.

As an alternative to using saccharide antigens from pneumococcus, the composition may include one or more polypeptide antigens. Genome sequences for several strains of pneumococcus are available [103,104] and can be subjected to reverse vaccinology [105-108] to identify suitable polypeptide antigens [109,110]. For example, the composition may include one or more of the following antigens: PhtA, PhtD, PhtB, PhtE, SpsA, LytB, LytC, LytA, Sp125, Sp101, Sp128 and Sp130, as defined in reference 111.

In some embodiments, the composition may include both saccharide and polypeptide antigens from pneumococcus. These may be used in simple admixture, or the pneumococcal saccharide antigen may be conjugated to a pneumococcal protein. Suitable carrier proteins for such embodiments include the antigens listed in the previous paragraph [111].

Pneumococcal antigens may be lyophilised *e.g.* together with meningococcal and/or Hib antigens.

Covalent conjugation

Capsular saccharides in compositions of the invention will usually be conjugated to carrier protein(s). In general, conjugation enhances the immunogenicity of saccharides as it converts them from T-independent antigens to T-dependent antigens, thus allowing priming for immunological memory. Conjugation is particularly useful for paediatric vaccines and is a well known technique [*e.g.* reviewed in refs. 112 and 88-96].

Preferred carrier proteins are bacterial toxins or toxoids, such as diphtheria toxoid or tetanus toxoid. The CRM_{197} mutant diphtheria toxin [79,113,114] is particularly preferred. Other suitable carrier proteins include the *N.meningitidis* outer membrane protein [68], synthetic peptides [69,70], heat shock proteins [71,72], pertussis proteins [73,74], cytokines [76], lymphokines [76], hormones [76], growth factors [76], artificial proteins comprising multiple human $CD4^+$ T cell epitopes from various pathogen-derived antigens [115], protein D from *H.influenzae* [75,116], pneumococcal surface protein PspA [117], iron-uptake proteins [78], toxin A or B from *C.difficile* [77], *etc.* Preferred carriers are diphtheria toxoid, tetanus toxoid, *H.influenzae* protein D, and CRM_{197}.

Within a composition of the invention, it is possible to use more than one carrier protein *e.g.* to reduce the risk of carrier suppression. Thus different carrier proteins can be used for different serogroups *e.g.* serogroup A saccharides might be conjugated to CRM_{197} while serogroup C saccharides might be conjugated to tetanus toxoid. It is also possible to use more than one carrier protein for a particular saccharide antigen *e.g.* serogroup A saccharides might be in two groups, with some conjugated to CRM_{197} and others conjugated to tetanus toxoid. In general, however, it is preferred to use the same carrier protein for all saccharides.

A single carrier protein might carry more than one saccharide antigen [118]. For example, a single carrier protein might have conjugated to it saccharides from serogroups A and C. To achieve this goal, saccharides can be mixed prior to the conjugation reaction. In general, however, it is preferred to have separate conjugates for each serogroup.

Conjugates with a saccharide:protein ratio (w/w) of between 1:5 (*i.e.* excess protein) and 5:1 (*i.e.* excess saccharide) are preferred. Ratios between 1:2 and 5:1 are preferred, as are ratios between 1:1.25 and 1:2.5 are more preferred. Excess carrier protein is preferred for MenA and MenC.

Conjugates may be used in conjunction with free carrier protein [119]. When a given carrier protein is present in both free and conjugated form in a composition of the invention, the unconjugated form is preferably no more than 5% of the total amount of the carrier protein in the composition as a whole, and more preferably present at less than 2% by weight.

Any suitable conjugation reaction can be used, with any suitable linker where necessary.

The saccharide will typically be activated or functionalised prior to conjugation. Activation may involve, for example, cyanylating reagents such as CDAP (*e.g.* 1-cyano-4-dimethylamino pyridinium tetrafluoroborate [120,121,*etc.*]). Other suitable techniques use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC, TSTU; see also the introduction to reference 94).

Linkages via a linker group may be made using any known procedure, for example, the procedures described in references 122 and 123. One type of linkage involves reductive amination of the polysaccharide, coupling the resulting amino group with one end of an adipic acid linker group, and then coupling a protein to the other end of the adipic acid linker group [92,124,125]. Other linkers include B-propionamido [126], nitrophenyl-ethylamine [127], haloacyl halides [128], glycosidic linkages [129], 6-aminocaproic acid [130], ADH [131], C_4 to C_{12} moieties [132] *etc.* As an alternative to using a linker, direct linkage can be used. Direct linkages to the protein may comprise oxidation of the polysaccharide followed by reductive amination with the protein, as described in, for example, references 133 and 134.

A process involving the introduction of amino groups into the saccharide (*e.g.* by replacing terminal =O groups with $-NH_2$) followed by derivatisation with an adipic diester (*e.g.* adipic acid N-hydroxysuccinimido diester) and reaction with carrier protein is preferred. Another preferred reaction uses CDAP activation with a protein D carrier *e.g.* for MenA or MenC.

After conjugation, free and conjugated saccharides can be separated. There are many suitable methods, including hydrophobic chromatography, tangential ultrafiltration, diafiltration *etc.* [see also refs. 135 & 136, *etc.*].

Where the composition of the invention includes a conjugated oligosaccharide, it is preferred that oligosaccharide preparation precedes conjugation.

Outer membrane vesicles

It is preferred that compositions of the invention should not include complex or undefined mixtures of antigens, which are typical characteristics of OMVs. However, one way in which the invention can be applied to OMVs is where OMVs are to be administered in a multiple dose schedule.

Where more than one OMV dose is to be administered, each dose may be supplemented (either by adding the purified protein or by expression of the protein within the bacteria from which the OMVs are derived) by one of the first protein, second protein or third protein as defined above. Preferably different doses are supplemented with different NMB1870 variants. In a three dose OMV schedule, for example, each dose could contain a different one of the first protein, second protein and third protein such that, after receiving three doses of OMVs, all three variants have been received. In a two dose OMV schedule, one variant could be used per OMV dose (thus omitting one variant), or one or both OMV doses could be supplemented with more than one variant in order to give coverage with all three variants. In preferred embodiments, there are three OMV doses, and each of the three OMV doses contains three different genetically-engineered vesicle populations each displaying three subtypes, thereby giving nine different subtypes in all.

This approach may be used in general to improve preparations of *N.meningitidis* serogroup B microvesicles [137], 'native OMVs' [138], blebs or outer membrane vesicles [*e.g.* refs. 139 to 144, *etc.*]. These may be prepared from bacteria which have been genetically manipulated [145-148] *e.g.* to increase immunogenicity (*e.g.* hyper-express immunogens), to reduce toxicity, to inhibit capsular polysaccharide synthesis, to down-regulate PorA expression, *etc.* They may be prepared from hyperblebbing strains [149-152]. Vesicles from a non-pathogenic *Neisseria* may be included [153]. OMVs may be prepared without the use of detergents [154,155]. They may express non-Neisserial proteins on their surface [156]. They may be LPS-depleted. They may be mixed with recombinant antigens [139,157]. Vesicles from bacteria with different class I outer membrane protein subtypes may be used *e.g.* six different subtypes [158,159] using two different genetically-engineered vesicle populations each displaying three subtypes, or nine different subtypes using three different genetically-engineered vesicle populations each displaying three subtypes, *etc.* Useful subtypes include: P1.7,16; P1.5-1,2-2; P1.19,15-1; P1.5-2,10; P1.12-1,13; P1.7-2,4; P1.22,14; P1.7-1,1; P1.18-1,3,6.

It is also possible, of course, to supplement vesicle preparations with two or three different variants.

Immunisation

The composition of the invention is preferably an immunogenic composition, and the invention provides an immunogenic composition of the invention for use as a medicament.

The invention also provides a method for raising an antibody response in a mammal, comprising administering an immunogenic composition of the invention to the mammal. The antibody response is preferably a protective and/or bactericidal antibody response.

The invention also provides a method for protecting a mammal against a Neisserial (*e.g.* meningococcal) infection, comprising administering to the mammal an immunogenic composition of the invention.

The invention also provides the use of at least two of antigens (a), (b) and (c) as defined above in the manufacture of a medicament for preventing Neisserial (*e.g.* meningococcal) infection in a mammal.

The mammal is preferably a human. The human may be an adult or, preferably, a child.

Immunogenic compositions of the invention may be used therapeutically (*i.e.* to treat an existing infection) or prophylactically (*i.e.* to prevent future infection).

The uses and methods are particularly useful for preventing/treating diseases including, but not limited to, meningitis (particularly bacterial meningitis) and bacteremia.

Efficacy of therapeutic treatment can be tested by monitoring Neisserial infection after administration of the composition of the invention. Efficacy of prophylactic treatment can be tested by monitoring immune responses against NMB1870 after administration of the composition. Immunogenicity of compositions of the invention can be determined by administering them to test subjects (*e.g.* children 12-16 months age, or animal models [160]) and then determining standard parameters including serum bactericidal antibodies (SBA) and ELISA titres (GMT) of total and high-avidity anti-capsule IgG. These immune responses will generally be determined around 4 weeks after administration of the composition, and compared to values determined before administration of the composition. A SBA increase of at least 4-fold or 8-fold is preferred. Where more than one dose of the composition is administered, more than one post-administration determination may be made.

Preferred compositions of the invention can confer an antibody titre in a patient that is superior to the criterion for seroprotection for each antigenic component for an acceptable percentage of human subjects. Antigens with an associated antibody titre above which a host is considered to be seroconverted against the antigen are well known, and such titres are published by organisations such as WHO. Preferably more than 80% of a statistically significant sample of subjects is seroconverted, more preferably more than 90%, still more preferably more than 93% and most preferably 96-100%.

Compositions of the invention will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection (*e.g.* subcutaneously, intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or by rectal, oral, vaginal, topical, transdermal, intranasal, ocular, aural, pulmonary or other mucosal administration. Intramuscular administration to the thigh or the upper arm is preferred. Injection may be via a needle (*e.g.* a hypodermic needle), but needle-free injection may alternatively be used. A typical intramuscular dose is 0.5 ml.

The invention may be used to elicit systemic and/or mucosal immunity.

Dosage treatment can be a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. A primary dose

schedule may be followed by a booster dose schedule. Suitable timing between priming doses (*e.g.* between 4-16 weeks), and between priming and boosting, can be routinely determined.

The immunogenic composition of the invention will generally include a pharmaceutically acceptable carrier, which can be any substance that does not itself induce the production of antibodies harmful to the patient receiving the composition, and which can be administered without undue toxicity. Suitable carriers can be large, slowly-metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Pharmaceutically acceptable carriers can include liquids such as water, saline, glycerol and ethanol. Auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, can also be present in such vehicles. Liposomes are suitable carriers. A thorough discussion of pharmaceutical carriers is available in ref. 161.

Neisserial infections affect various areas of the body and so the compositions of the invention may be prepared in various forms. For example, the compositions may be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The composition may be prepared for topical administration *e.g.* as an ointment, cream or powder. The composition be prepared for oral administration *e.g.* as a tablet or capsule, or as a syrup (optionally flavoured). The composition may be prepared for pulmonary administration *e.g.* as an inhaler, using a fine powder or a spray. The composition may be prepared as a suppository or pessary. The composition may be prepared for nasal, aural or ocular administration *e.g.* as drops.

The composition is preferably sterile. It is preferably pyrogen-free. It is preferably buffered *e.g.* at between pH 6 and pH 8, generally around pH 7. Where a composition comprises an aluminium hydroxide salt, it is preferred to use a histidine buffer [162]. Compositions of the invention may be isotonic with respect to humans.

Immunogenic compositions comprise an immunologically effective amount of immunogen, as well as any other of other specified components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (*e.g.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Dosage treatment may be a single dose schedule or a multiple dose schedule (*e.g.* including booster doses). The composition may be administered in conjunction with other immunoregulatory agents.

An immunogenic composition will generally include an adjuvant. Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (A) MF59 (5% Squalene, 0.5%

Tween 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer) [see Chapter 10 of ref. 163; see also ref. 164]; (B) microparticles (*i.e.* a particle of ~100nm to ~150μm in diameter, more preferably ~200nm to ~30μm in diameter, and most preferably ~500nm to ~10μm in diameter) formed from materials that are biodegradable and non-toxic (*e.g.* a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone *etc.*), with poly(lactide-co-glycolide) being preferred ('PLG'), optionally havnig a charged surface (*e.g.* by adding a cationic, anionic, or nonionic detergent such as SDS (negative) or CTAB (positive) [*e.g.* refs. 165 & 166]); (C) liposomes [see Chapters 13 and 14 of ref. 163]; (D) ISCOMs [see Chapter 23 of ref. 163] , which may be devoid of additional detergent [167]; (E) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion [see Chapter 12 of ref. 163]; (F) Ribi™ adjuvant system (RAS), (Ribi Immunochem) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (G) saponin adjuvants, such as QuilA or QS21 [see Chapter 22 of ref. 163], also known as Stimulon™; (H) chitosan [*e.g.* 168]; (I) complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA); (J) cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, *etc.*), interferons (*e.g.* interferon-γ), macrophage colony stimulating factor, tumor necrosis factor, *etc.* [see Chapters 27 & 28 of ref. 163], RC529; (K) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol) [169]; (L) monophosphoryl lipid A (MPL) or 3-O-deacylated MPL (3dMPL) [*e.g.* chapter 21 of ref. 163]; (M) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions [170]; (N) oligonucleotides comprising CpG motifs [171] *i.e.* containing at least one CG dinucleotide, with 5-methylcytosine optionally being used in place of cytosine; (O) a polyoxyethylene ether or a polyoxyethylene ester [172]; (P) a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol [173] or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol [174]; (Q) an immunostimulatory oligonucleotide (*e.g.* a CpG oligonucleotide) and a saponin [175]; (R) an immunostimulant and a particle of metal salt [176]; (S) a saponin and an oil-in-water emulsion [177]; (T) *E.coli* heat-labile enterotoxin ("LT"), or detoxified mutants thereof, such as the K63 or R72 mutants [*e.g.* Chapter 5 of ref. 38]; (U) cholera toxin ("CT"), or detoxified mutants thereof [*e.g.* Chapter 5 of ref. 38]; (V) double-stranded RNA; (W) aluminium salts, such as aluminium hydroxides (including oxyhydroxides), aluminium phosphates (including hydroxyphosphates), aluminium sulfate, *etc* [Chapters 8 & 9 in ref. 163] or calcium salts, such as calcium phosphate; and (X) other substances that act as immunostimulating agents to enhance the effectiveness of the composition [*e.g.* see Chapter 7 of ref. 163]. Aluminium salts (aluminium phosphates and particularly hydroxyphosphates, and/or hydroxides and particularly oxyhydroxide) and MF59 are preferred adjuvants for parenteral immunisation. Toxin mutants are preferred mucosal adjuvants. QS21 is another useful adjuvant for NMB1870, which may be used alone or in combination with any of (A) to (X) *e.g.* with an aluminium salt.

Muramyl peptides include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE), *etc.*

Protein expression

Bacterial expression techniques are known in the art. A bacterial promoter is any DNA sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of a coding sequence (*e.g.* structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A bacterial promoter may also have a second domain called an operator, that may overlap an adjacent RNA polymerase binding site at which RNA synthesis begins. The operator permits negative regulated (inducible) transcription, as a gene repressor protein may bind the operator and thereby inhibit transcription of a specific gene. Constitutive expression may occur in the absence of negative regulatory elements, such as the operator. In addition, positive regulation may be achieved by a gene activator protein binding sequence, which, if present is usually proximal (5') to the RNA polymerase binding sequence. An example of a gene activator protein is the catabolite activator protein (CAP), which helps initiate transcription of the lac operon in Escherichia coli (E. coli) [Raibaud *et al.* (1984) *Annu. Rev. Genet. 18*:173]. Regulated expression may therefore be either positive or negative, thereby either enhancing or reducing transcription.

Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) [Chang *et al.* (1977) *Nature 198*:1056], and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*) [Goeddel *et al.* (1980) *Nuc. Acids Res. 8*:4057; Yelverton *et al.* (1981) *Nucl. Acids Res. 9*:731; US patent 4,738,921; EP-A-0036776 and EP-A-0121775]. The β-lactamase (*bla*) promoter system [Weissmann (1981) "The cloning of interferon and other mistakes." In *Interferon 3* (ed. I. Gresser)], bacteriophage lambda PL [Shimatake *et al.* (1981) *Nature 292*:128] and T5 [US patent 4,689,406] promoter systems also provide useful promoter sequences. Another promoter of interest is an inducible arabinose promoter (pBAD).

In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or bacteriophage promoter may be joined with the operon sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter [US patent 4,551,433]. For example, the *tac* promoter is a hybrid *trp-lac* promoter comprised of both *trp* promoter and *lac* operon sequences that is regulated by the *lac* repressor [Amann *et al.* (1983) *Gene 25*:167; de Boer *et al.* (1983) *Proc. Natl. Acad. Sci. 80*:21]. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes. The bacteriophage T7 RNA polymerase/promoter system is an example of a coupled promoter system [Studier *et al.* (1986) *J. Mol. Biol. 189*:113; Tabor *et al.* (1985) *Proc Natl. Acad. Sci. 82*:1074]. In addition, a hybrid promoter can also be comprised of a bacteriophage promoter and an *E. coli* operator region (EPO-A-0 267 851).

In addition to a functioning promoter sequence, an efficient ribosome binding site is also useful for the expression of foreign genes in prokaryotes. In *E. coli*, the ribosome binding site is called the Shine-Dalgarno (SD) sequence and

includes an initiation codon (ATG) and a sequence 3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon [Shine *et al.* (1975) *Nature 254*:34]. The SD sequence is thought to promote binding of mRNA to the ribosome by the pairing of bases between the SD sequence and the 3' and of *E. coli* 16S rRNA [Steitz *et al.* (1979) "Genetic signals and nucleotide sequences in messenger RNA." In *Biological Regulation and Development: Gene Expression* (ed. R.F. Goldberger)]. To express eukaryotic genes and prokaryotic genes with weak ribosome-binding site [Sambrook *et al.* (1989) "Expression of cloned genes in Escherichia coli." In *Molecular Cloning: A Laboratory Manual*].

A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide or by either *in vivo* on *in vitro* incubation with a bacterial methionine N-terminal peptidase (EP-A-0219237).

Usually, transcription termination sequences recognized by bacteria are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Transcription termination sequences frequently include DNA sequences of about 50 nucleotides capable of forming stem loop structures that aid in terminating transcription. Examples include transcription termination sequences derived from genes with strong promoters, such as the *trp* gene in *E. coli* as well as other biosynthetic genes.

Usually, the above described components, comprising a promoter, signal sequence (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (*e.g.* plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will have a replication system, thus allowing it to be maintained in a prokaryotic host either for expression or for cloning and amplification. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably contain at least about 10, and more preferably at least about 20 plasmids. Either a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host.

Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to the bacterial chromosome that allows the vector to integrate. Integrations appear to result from recombinations between homologous DNA in the vector and the bacterial chromosome. For example, integrating vectors constructed with DNA from various Bacillus strains integrate into the Bacillus chromosome (EP-A-0127328). Integrating vectors may also be comprised of bacteriophage or transposon sequences.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of bacterial strains that have been transformed. Selectable markers can be expressed in the bacterial host and may include genes which render bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin (neomycin), and tetracycline [Davies *et al.* (1978) *Annu. Rev. Microbiol. 32*:469]. Selectable markers may also include biosynthetic genes, such as those in the histidine, tryptophan, and leucine biosynthetic pathways.

Alternatively, some of the above described components can be put together in transformation vectors. Transformation vectors are usually comprised of a selectable market that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extra-chromosomal replicons or integrating vectors, have been developed for transformation into many bacteria. For example, expression vectors have been developed for, *inter alia*, the following bacteria: Bacillus subtilis [Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA 79*:5582; EP-A-0 036 259 and EP-A-0 063 953; WO 84/04541], Escherichia coli [Shimatake *et al.* (1981) *Nature 292*:128; Amann *et al.* (1985) *Gene 40*:183; Studier *et al.* (1986) *J. Mol. Biol. 189*:113; EP-A-0 036 776,EP-A-0 136 829 and EP-A-0 136 907], Streptococcus cremoris [Powell *et al.* (1988) *Appl. Environ. Microbiol. 54*:655]; Streptococcus lividans [Powell *et al.* (1988) *Appl. Environ. Microbiol. 54*:655], Streptomyces lividans [US patent 4,745,056].

Methods of introducing exogenous DNA into bacterial hosts are well-known in the art, and usually include either the transformation of bacteria treated with $CaCl_2$ or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation. Transformation procedures usually vary with the bacterial species to be transformed. See *e.g.* [Masson *et al.* (1989) *FEMS Microbiol. Lett. 60*:273; Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA 79*:5582; EP-A-0 036 259 and EP-A-0 063 953; WO 84/04541, Bacillus], [Miller *et al.* (1988) *Proc. Natl. Acad. Sci. 85*:856; Wang *et al.* (1990) *J. Bacteriol. 172*:949, Campylobacter], [Cohen *et al.* (1973) *Proc. Natl. Acad. Sci. 69*:2110; Dower *et al.* (1988) *Nucleic Acids Res. 16*:6127; Kushner (1978) "An improved method for transformation of Escherichia coli with ColE1-derived plasmids. In *Genetic Engineering: Proceedings of the International Symposium on Genetic Engineering* (eds. H.W. Boyer and S. Nicosia); Mandel *et al.* (1970) *J. Mol. Biol. 53*:159; Taketo (1988) *Biochim. Biophys. Acta 949*:318; Escherichia], [Chassy *et al.* (1987) *FEMS Microbiol. Lett. 44*:173 Lactobacillus]; [Fiedler *et al.* (1988) *Anal. Biochem 170*:38, Pseudomonas]; [Augustin *et al.* (1990) *FEMS Microbiol. Lett. 66*:203, Staphylococcus], [Barany *et al.* (1980) *J. Bacteriol. 144*:698; Harlander (1987) "Transformation of Streptococcus lactis by electroporation, in: *Streptococcal Genetics* (ed. J. Ferretti and R. Curtiss III); Perry *et al.* (1981) *Infect. Immun. 32*:1295; Powell *et al.* (1988) *Appl. Environ. Microbiol. 54*:655; Somkuti *et al.* (1987) *Proc. 4th Evr. Cong. Biotechnology 1*:412, Streptococcus].

Disclaimers

The invention preferably excludes: (a) amino acid and nucleic acid sequences available in public sequence databases (*e.g.* GenBank or GENESEQ) prior to 22nd November 2002; (b) amino acid and nucleic acid sequences disclosed in patent applications having a filing date or, where applicable, a priority date prior to 22nd November 2002. In particular, SEQ ID entries in the any of the references cited herein may be excluded *e.g.* reference 13.

General

The term "comprising" means "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term "about" in relation to a numerical value *x* means, for example, $x \pm 10\%$.

The word "substantially" does not exclude "completely" *e.g.* a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

'Sequence identity' is preferably determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters *gap open penalty=12* and *gap extension penalty=1*.

After serogroup, meningococcal classification includes serotype, serosubtype and then immunotype, and the standard nomenclature lists serogroup, serotype, serosubtype, and immunotype, each separated by a colon *e.g.* B:4:P1.15:L3,7,9. Within serogroup B, some lineages cause disease often (hyperinvasive), some lineages cause more severe forms of disease than others (hypervirulent), and others rarely cause disease at all. Seven hypervirulent lineages are recognised, namely subgroups I, III and IV-1, ET-5 complex, ET-37 complex, A4 cluster and lineage 3. These have been defined by multilocus enzyme electrophoresis (MLEE), but multilocus sequence typing (MLST) has also been used to classify meningococci [ref. 16]. The four main hypervirulent clusters are ST32, ST44, ST8 and ST11 complexes.

The term "alkyl" refers to alkyl groups in both straight and branched forms, The alkyl group may be interrupted with 1, 2 or 3 heteroatoms selected from -O-, -NH- or -S-. The alkyl group may also be interrupted with 1, 2 or 3 double and/or triple bonds. However, the term "alkyl" usually refers to alkyl groups having no heteroatom interruptions or double or triple bond interruptions. Where reference is made to C_{1-12} alkyl, it is meant the alkyl group may contain any number of carbon atoms between 1 and 12 (*e.g.* C_1, C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}). Similarly, where reference is made to C_{1-6} alkyl, it is meant the alkyl group may contain any number of carbon atoms between 1 and 6 (*e.g.* C_1, C_2, C_3, C_4, C_5, C_6).

The term "cycloalkyl" includes cycloalkyl, polycycloalkyl, and cycloalkenyl groups, as well as combinations of these with alkyl groups, such as cycloalkylalkyl groups. The cycloalkyl group may be interrupted with 1, 2 or 3 heteroatoms selected from -O-, -NH- or -S-. However, the term "cycloalkyl" usually refers to cycloalkyl groups having no heteroatom interruptions Examples of cycloalkyl groups include cyclopentyl, cyclohexyl, cyclohexenyl, cyclohexylmethyl and adamantyl groups. Where reference is made to C_{3-12} cycloalkyl, it is meant that the cycloalkyl group may contain any number of carbon atoms between 3 and 12 (*e.g.* C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}).

The term "aryl" refers to an aromatic group, such as phenyl or naphthyl. Where reference is made to C_{5-12} aryl, it is meant that the aryl group may contain any number of carbon atoms between 5 and 12 (*e.g.* C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}).

The term "C_{5-12} aryl-C_{1-6} alkyl" refers to groups such as benzyl, phenylethyl and naphthylmethyl.

Nitrogen protecting groups include silyl groups (such as TMS, TES, TBS, TIPS), acyl derivatives (such as phthalimides, trifluoroacetamides, methoxycarbonyl, ethoxycarbonyl, t-butoxycarbonyl (Boc), benzyloxycarbonyl (Z or Cbz), 9-fluorenylmethoxycarbonyl (Fmoc), 2-(trimethylsilyl)ethoxy

carbonyl, 2,2,2-trichloroethoxycarbonyl (Troc)), sulfonyl derivatives (such as β-trimethylsilylethanesulfonyl (SES)), sulfenyl derivatives, C_{1-12} alkyl, benzyl, benzhydryl, trityl, 9-phenylfluorenyl *etc.* A preferred nitrogen protecting group is Fmoc.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the nucleotide sequence of the upstream region of NMB1870.

Figure 2 is a schematic representation of the structure of TbpB proteins and of antigens NMB2132 and NMB1870. The leader peptides and proximal glycine-rich regions are indicated. Five conserved boxes are indicated by different motifs and their positions are mapped on the protein sequence.

Figure 3 shows the increase in NMB1870 levels in *N.meningitidis* MC58 during the growth curve. Figure 4 shows the increase in NMB1870 levels in the supernatant over the same period. PorA levels and NMB1380 levels do not increase. Numbers above lanes refer to OD_{620nm} of the culture. KO indicates a NMB1870 knockout mutant of MC58, and Wcl stands for the whole cell lysate control.

Figure 5 shows OMVs probed with anti-NMB1870.

Figure 6 shows FACS analysis of encapsulated MC58 or a non-encapsulated mutant MC58 using anti-NMB1870.

Figure 7 is a western blot of a gradient SDS-PAGE gel loaded with total cell lysates of high (lanes 1 & 2), intermediate (3 & 4) and low (5 & 6) NMB1870 expressers. Lane 7 contains a MC58 NMB1870 knockout. Lanes are: (1) MC58; (2) H44/76; (3) NZ394/98; (4) 961-5945; (5) 67/00; (6) M1239; (7) MC58Δnmb1870.

Figure 8 shows FACS and bactericidal titres for each of a high, intermediate and low expresser, and also for the NMB1870 knockout. The intermediate and low expresser have identical NMB1870 amino acid sequences, with a 91.6% match to MC58.

Figure 9 is a dendrogram showing the strain clustering according to NMB1870 protein distances. The labels '1', '2' and '3' indicate the three variants. Numbers in square brackets indicate the number of strains with identical sequence present in each branch of the dendrogram. Hypervirulent lineages are indicated, followed by the number of strains when this is different from the total number. Serogroups other than B are also shown. The three type strains (MC58, 961-5965 and M1239) and the other strains used in the serological analysis are within circles.

Figure 10 is a sequence alignment of variant 1 (MC58), variant 2 (961-5945), and variant 3 (M1239). Amino acid numbers initiate from the cysteine predicted to be the first amino acid of the mature protein. Grey and black backgrounds indicate conserved and identical residues, respectively.

Figure 11 shows FACS analysis of sera against variant 1 (first row), variant 2 (second row), and variant 3 (third row), using the type strain of variant 1 (MC58), variant 2 (961-5945) and variant 3 (M1239). Control sera against the capsular polysaccharide is shown in row 4 (monoclonal antibody Seam3). Control serum against a cytoplasmic protein is shown in row 5 (anti-NMB1380). Row 6 contains the knock out mutants (KO) of each type strain, probed with the homologous antiserum.

Figures 12 (variant 1), 13 (variant 2) and 14 (variant 3) show dendrograms for the three separate variants of NMB1870, classified by multilocus sequence types (ST).

MODES FOR CARRYING OUT THE INVENTION

NMB1870 in serogroup B strain MC58 — identification of the start codon

The NMB1870 gene was identified in the genome sequences of MenB and MenA published by The Institute for Genomic Research (TIGR) and Sanger Center, respectively [2,4; NMB1870 and NMA0586]. However, there is a discrepancy over the position of the ATG start codon as the MenB start codon is 120bp upstream of the MenA start codon. In contrast to both prior art annotations, the present invention places the start codon as a GTG codon which is downstream of the prior art start codons (18bp downstream for MenA, 138bp for MenB) and agrees with reference 8.

As shown in Figure 1, the GTG start (+1) is consistent with the presence of a correctly spaced ribosome-binding site and with the prediction of the lipoprotein signature. The prior art TIGR MenB start codon is shown in a box, and the Sanger MenA start codon is in a circle. Inverted repeats are shown by horizontal arrows.

NMB1870 is a monocistronic gene located 157 bases downstream the stop codon of the fructose-bisphosphate aldolase gene nmb1869. In MenA Z2491 the overall organisation is similar, but 31 base pairs upstream from the GTG starting codon there is an insertion of 186 nucleotides which are homologous to an internal repeat region of IS1106 and are flanked by two 16 base pairs inverted repeats. A putative ribosome binding site (shaded) is present 8 bp upstream from the GTG starting codon. A fur box (11/19 matches with the *E.coli* fur box consensus [178]; SEQ ID NO^{S}: 74 & 75) is located 35bp upstream of the start codon, as predicted by GCG FindPatterns starting from SEQ ID NO: 75 and allowing a maximum of nine mismatches. Putative promoter sequences were also detected.

The GCG Wisconsin Package suite (version 10.0) was used for computer sequence analysis of gene and protein sequences. The PSORT program [179] was used for localisation prediction. NMB1870 has the typical signature of a surface-exposed lipoprotein, characterised by a signal peptide with a lipo-box motif of the type -Leu-X-X-Cys-, where the Cysteine was followed by a Serine, an amino acid generally associated with outer membrane localisation of lipoproteins [180]. The lipo-box is lost in gonococcus due to a frame-shifting single base (G) insertion after MC58 nucleotide 36, with the correct reading frame being re-established by a 8bp insertion after position 73.

The mature MC58 protein is predicted to be a lipoprotein with a molecular weight of 26,964 Da and a pI of 7.96, and is characterised by the presence of four glycines downstream of the lipo-box motif.

Secondary structure prediction analysis using the PredictProtein software [181] indicates that NMB1870 is a globular protein mostly composed of beta sheets.

Sequence analysis

The PSI-BLAST algorithm was used for homology searches [182] using the non-redundant protein database. No homologous proteins were found by searching existing non-redundant prokaryotic and eukaryotic protein databases maintained at the NCBI site, including the human genome, suggesting that NMB1870 is specific for *Neisseria*. However, a domain with some homology (28% identity over 146 amino acids) was found with the C-terminal portion of the transferrin-binding protein TfbA of *Actinobacillus pleuropneumoniae* [183] (Figure 2). A closer look at this domain revealed homologies also with the transferrin-binding proteins from *N. meningitidis* [184], *H. influenzae* [185], *Moraxella catarrhalis* [186] and with the *N. meningitidis* surface antigen NMB2132, previously annotated as TbpB homologue [3].

To see if this sequence homology reflects a functional homology, recombinant NMB1870 (see below), human transferrin hTF (Sigma T-4132) and the mix of the two (final concentration of 7μM) were dialysed O/N in PBS at 4°C. Following dialysis 20μl of each protein and the mixture of them were loaded on a HPLC Superdex 200 PC 3.2/30 gel filtration column (Amersham) using PBS as running buffer [187]. Blue Dextran 2000 and the molecular weight standards ribonuclease A, chymotrypsin A, ovalbumin A, bovine serum albumin (Amersham) were used to calibrate the column. Gel filtration was performed using a Smart system with a flow rate of 0.04 ml/min and the eluted material monitored at 214 nm and 280 nm. (The NMB1870 retention volume was 1.68 ml and 1.47 ml for htf.) Fractions of 40μl were collected and analysed by SDS-PAGE. The MC58 recombinant transferrin-binding protein 2 (Tbp2) was used as positive control.

The recombinant protein failed to bind human transferrin *in vitro*.

The Fur box in the promoter suggests that the expression of NMB1870 may be regulated by iron. However, expression of the protein does not seem to increase in low iron conditions.

An interesting feature of the protein is the presence of a stretch of four glycines downstream from the lipidated cysteine. Three or more consecutive glycines downstream from a lipidated cysteine are present also in other five lipoproteins in *N.meningitidis*, namely the transferrin-binding protein B (TbpB), the outer membrane component of an ABC transporter NMB0623, the hypothetical protein NMB1047, the TbpB homologue NMB2132, and the AspA lipoprotein [188]. In none of these proteins the poly-glycine stretch is encoded by a poly-G tract, suggesting that this feature is not used to generate antigenic modulation.

A search for lipoproteins with a glycine-rich region was carried out on 22 complete genomic sequences retrieved at the NCBI site [189] using FindPatterns. The search retrieved 29 lipoproteins in some but not all bacterial species. The organisms with this type of lipoproteins include both Gram-negative and Gram-positive bacteria, including *Haemophilus influenzae*, *Enterococcus fecalis*, *Mycobacterium tuberculosis*, *Lysteria monocytogenes* and *Staphylococcus aureus*, while others such as *E.coli*, *Bacillus subtilis*, *Helicobacter pylori*, *Streptococcus pneumoniae*, *S.pyogenes* and *Vibrio cholerae* have none. Most of the lipoproteins with this signature belong to ABC transporters, followed by proteins of unknown function. Although this common feature in the primary structure

suggests a common role for the glycine repeats, so far, the function is unknown. However, it may serve to guide the lipoproteins to a specific pathway of secretion and surface localisation [190].

Sequencing for other strains

70 strains representative of the genetic and geographic diversity of the *N.meningitidis* population were selected for further investigation of NMB1870. Strains derive from 19 different countries, 73% belong to serogroup B, and 32 were isolated in the last five years. The strain panel mostly includes serogroup B strains, a few strains of serogroups A, C, Y, W-135 and Z, and one strain each of *N.gonorrhoeae* and *N.cinerea.* Strains are disclosed in more detail in references 191 & 192. Some strains are available from the ATCC (*e.g.* strain MC58 is available under reference BAA-335).

The NMB1870 gene was amplified using primers external to the coding sequence (A1, SEQ ID 55; and B2, SEQ ID 56). About 10 ng of chromosomal DNA was used as template for the amplification. PCR conditions were: 30 cycles, 94 °C for 40" 58 °C for 40" 68 °C for 40". PCR fragment were purified by the Qiagen QIAquick PCR Purification Kit, and submitted to sequence analysis, which was performed using an ABI 377 Automatic Sequencer. Sequencing was performed using primers A1, B2, 22 (SEQ ID 57) and 32 (SEQ ID 58).

The gene was detected by PCR in all 70 *Neisseria* strains. In *N.lactamica* a band could be detected by Western blotting, but the gene could not be amplified.

The nucleotide sequence of the gene was determined in all 70 strains. A total of 23 different protein sequences were encoded (SEQ ID NO^S 1 to 23). Computer analysis of these 23 sequences, using Kimura and Jukes-Cantor algorithm, divided them into three variants (Figure 9). The dendrogram was obtained starting from the multiple sequence alignment of NMB1870 protein sequences (PileUP) using the Protein Sequence Parsimony Method (ProtPars), a program available within the Phylogeny Inference Package (Phylip), and confirmed by the GCG program Distances, using the Kimura and Jukes-Cantor algorithms.

The NMB1870 sequences from 100 further strains were determined. Many of these were identical to one of SEQ ID NO^S 1 to 23, but 19 further unique sequences are given as SEQ ID NO^S 140 to 158.

Figures 12-14 show dendrograms of the various sequences, classified by ST multilocus sequence types. Within variant 1 (Figure 12) the reference strain is MC58, with the lowest sequence identity to the reference being 89.4% against an average of 93.7%. Within variant 2 (Figure 13) the reference strain is 2996 and sequences extend down to 93.4% identity (average 96.3%). Within variant 3 (Figur 14) the lowest identity to reference strain M1239 is 94.7% (average 95.8%). ST32cpx is the most homogeneous hypervirulent cluster, harbouring only one NMB8170 sequence from variant 1 (also also only one form of NMB1343 and of NadA). Most ST44cpx strains harbour variant 1 (several different sequences) of NMB1870, with some having variant 3 (single sequence). These data suggest that ST32cpx is closer to ST44cpx, as compared to other clusters, which matches data based on porA genotype (class III). ST11 and ST8 complexes are mostly represented by different sequences within variant 2 of NMB1870, suggesting that these complexes are closer together, as compared to other

clusters, and matching the porA genotype (class II). ST11cpx harbours all three variants, indicating that it is the most diverse hypervirulent cluster out of the four.

Strains MC58, 961-5945 and M1239 were arbitrarily selected as type strains for variants 1, 2 and 3, respectively. The sequence diversity between the three type strains is shown in Figure 10. Amino acid identity was 74.1% between variant 1 and variant 2, 62.8% between variant 1 and variant 3, and 84.7% between variant 2 and variant 3. Sequences within each variant were well-conserved, the most distant showing 91.6%, 93.4% and 93.2% identity to their type strains, respectively. *N.cinerea* belongs to variant 1, and shares 96,7% homology with MC58. As shown in Figure 9, variant 1 harbours all strains from hypervirulent lineages ET-5, most lineage 3 strains, the serogroup A strains, two recent isolates of W-135 and one ET-37. Variant 2 harbours all strains from the hypervirulent complex A4, from serogroups Y and Z, one old W-135 isolate and five ET-37 strains. Variant 3 harbours four unique ST strains, one ET-37 strain, one lineage 3 strain and gonococcus.

The strains in each variant group, and their NMB1870 sequences, are as follows:

1	gb185 (sequence shared with ES14784, M.00.0243291) m4030 (sequence shared with M3812) m2197 m2937 iss1001 (sequence shared with NZ394/98, 67/00, 93/114, bz198, m1390, nge28, 14996, 65/96, ISS1120, S59058, ISS1017, ISS1043, ISS1026, ISS1102, ISS1106, ISS656, ISS678, ISS740, ISS749, ISS995, ISS845, ISS1167, ISS1157, ISS1182, M4717, M6094, D8273) lnp17592 (sequence shared with 00-241341, 00-241357, 2ND80. 2ND221, ISS1142) f6124 (sequence shared with 205900) m198/172 (sequence shared with bz133, gb149, nm008, nm092, ES14963, FN131218, S5902, S90307, M4105, ISS1180, FN131345) mc58 (sequence shared with 30/00, 39/99, 72/00, 95330, bz169, bz83, cu385, h44/76, m1590, m2934, m2969, m3370, m4215, m4318, n44/89, 14847, ES14898, lnp15709, lnp17391, lnp17503, FN131654, M3985, S590104, S9029, S9097, D8346, FN131682, ISS832, ISS648, ISS1067, ISS1071, ISS1159) FN131217 ES14933 GB0993 M6190 F19324 ISS1113 gb0345 (sequence shared with M1820, ISS1082) M0445 17 sequences, 98 strains
2	L93/4286 m2671 961-6945 (sequence shared with 2996, 96217, 312294, 11327, a22, ISS1141, ISS1173, ISS759, ISS743, ISS866, F17094, NMB, SWZ107) gb013 (sequence shared with e32, m1090, m4287, 66094, M3153, M4407, NGH36)

	860800 (sequence shared with 599) 95N477 (sequence shared with 90-18311, c11, m986, F370/85, M.00.0243143, ISS838, ISS839, ISS1092, M1569) 1000 (sequence shared with m1096, M2552, M4458, M5149, M6208) m3279 (sequence shared with bz232, dk353, m3697, ngh38, M5258, D8221) MK82 8047 C4678 ISS1133 NG6/88 M0579 F16325 15 sequences, 56 strains
3	16889 m3813 m1239 ngp165 gb355 (sequence shared with m3369, D8300, gb0364, M2441) gb988 [fa1090 gonococcus] 7 sequences, 11 strains

NB: the abbreviation "gb" at the beginning of a strain name means "M.01.0240".

SEQ ID NO^{S} 139 (strain 220173i), 140 (strains gb101 & ISS908) and 141 (strain nge31) are distant from these three variants (as is, to a lesser degree, strain m3813).

Within variant 1, the strain lnp17592 sequence (also seen in strains 00-241341, 00-241357, 2ND80. 2ND221 & ISS1142) is seen in the W-135 Haji serogroup. Within the Haji strains, the NadA sequence (SEQ ID NO: 143) is a recombination between alleles 2 and 3 [191,192].

***Cloning, expression & purification in* E.coli**

NMB1870 genes were amplified by PCR from the genome of *N.meningitidis* MC58, 961-5945 and M1239 strains. Forward and reverse primers were designed in order to amplify the nmb1870 coding sequence devoid of the sequence coding for the putative leader peptide. M1239 and 961-5945 variants were found not to be expressible in *E.coli*. They were therefore expressed by adding to the N-terminal the sequence SEQ ID NO: 46 that is present in the gonococcus protein but absent in the meningococcus counterpart. Oligonucleotides used for the amplification were as follows:

Strain	Forward	Reverse
MC58	CGCGGATCCCATATG**GTCGCCGCCGACATCG** ('For1'; SEQ ID 47)	CCCGCTCGAG**TTGCTTGGCGGCAAGGC** ('Rev1'; SEQ ID 48)
961/5945	CGCGGATCCCATATG*GGCCCTGATTCTGACCGCCTGCAGCAGC* **GGAGGGTCGCCGCCGACATCGG** ('For2'; SEQ ID 49)	CCCGCTCGAG**CTGTTTGCCGGCGATGCC** ('Rev2'; SEQ ID 50)
M1239	CGCGGATCCCATATG*GGCCCTGATTCTGACCGCC TGCAGCAGCGGAGG***GGAGGGGGTGGTGTCGC** ('For3'; SEQ ID 51)	GCCCAAGCTT**CTGTTTGCCGGCGATGCC** ('Rev3'; SEQ ID 52)

Restriction sites, corresponding to *Nde*I for the forward primers and *Xho*I (*Hin*dIII for M1239) for the reverse primers, are underlined. For the 961-5945 and M1239 forward primers, the gonococcus sequence moiety is reported in italics, and the meningococcal NMB1870 matching sequences are reported in bold.

PCR conditions in the case of primer combination For1/Rev1 were: denaturation at 94°C for 30", annealing at 57°C for 30", elongation at 68°C for 1 min (5 cycles), denaturation at 94°C for 30", annealing at 68°C for 30", elongation at 68°C for 1 min (30 cycles). In the case of primer combinations: For2/Rev2 and For3/Rev2 and For3/Rev3: 94°C for 30", 56°C for 30", 68°C for 1 min (5 cycles), 94°C for 30", 71°C for 30", 68°C for 1 min (30 cycles).

Full-length nmb1870 gene was amplified from the MC58 genome using the following primers: f-lFor (CGCGGATCCCATATGAATCGAACTGCCTTCTGCTGCC; SEQ ID 53) and f-lRev (CCCGCTCGAGTTATTGCTTGGCGGCAAGGC; SEQ ID 54) and the following conditions: 94°C for 30", 58°C for 30", 72°C for 1 min (30 cycles).

PCR were performed on approx. 10 ng of chromosomal DNA using High Fidelity Taq DNA Polymerase (Invitrogen). The PCR products were digested with *Nde*I and *Xho*I and cloned into the *Nde*I/*Xho*I sites of the pET-21b+ expression vector (Novagen).

Recombinant proteins were expressed as His-tag fusions in *E.coli* and purified by MCAC (Metal Chelating Affinity Chromatography), as previously described [3], and used to immunise mice to obtain antisera. *E.coli* DH5α was used for cloning work, and BL21(DE_3) was used for expression.

nmb1870 and siaD isogenic mutants

Isogenic knockout mutants in which the nmb1870 gene was truncated and replaced with an erythromycin antibiotic cassette, was prepared by transforming strains MC58, 961-5945 and M1239 with the plasmid *pBSΔnmb1870ERM*. This plasmid contains the erythromycin resistance gene within the nmb1870 upstream and downstream flanking regions of 500bp. These regions were amplified from MC58 genome using the following oligonucleotides Ufor GCTCTAGACCAGCCAGGCGCATAC (SEQ ID 59, *Xba*I site underlined); URev TCCCCCGGGGACGGCATTTTGTTTACAGG (SEQ ID 60, *Sma*I underlined); DFor TCCCCCGGGCGCCAAGCAATAACCATTG (SEQ ID 61, *Sma*I underlined) and Drev CCCGCTCGAGCAGCGTATCGAACCATGC (SEQ ID 62, *Xho*I underlined). A capsule deficient mutant was generated using the same approach. The *siaD* gene was deleted and replaced with *ermC* using the plasmid *pBSΔCapERM*. The upstream and downstream flanking regions of 1000 bp and 1056 bp, respectively, were amplified from MC58 genome using the following primers: UCapFor

GCTCTAGATTCTTTCCCAAGAACTCTC (SEQ ID 63, *Xba*I underlined); UcapRev TCCCCCGGGCCCGTATCATCCACCAC (SEQ ID 64, *Sma*I underlined); DCapFor TCCCCCGGGATCCACGCAAATACCCC (SEQ ID 65, *Sma*I underlined) and DCapRev CCCGCTCGAGATATAAGTGGAAGACGGA (SEQ ID 66, *Xho*I underlined). Amplified fragments were cloned into pBluescript and transformed into naturally competent *N.meningitidis* strain MC58. The mixture was spotted onto a GC agar plate, incubated for 6 hrs at 37°C, 5% CO_2 then diluted in PBS and spread on GC agar plates containing 5 μg/ml erythromycin. The deletion of the nmb1870 gene in the MC58Δnmb1870, 961-5945Δnmb1870 and M1239Δnmb1870 strains was confirmed by PCR; lack of NMB1870 expression was confirmed by Western blot analysis. The deletion of the *siaD* gene and the lack of capsule expression in the MC58ΔsiaD strain were confirmed by PCR and FACS, respectively.

Lipoproteins

To investigate lipidation of NMB1870, palmitate incorporation of recombinant *E.coli* BL21(DE_3) strain carrying the full-length nmb1870 gene was tested as described in reference 193.

Meningococcal strains MC58 and MC58Δnmb1870 were grown in GC medium and labeled with [9,10-^{3}H]-palmitic acid (Amersham). Cells from 5 ml culture were lysed by boiling for 10 min and centrifuged at 13,000 rpm. The supernatants were precipitated with TCA and washed twice with cold acetone. Proteins were suspended in 50 μl of 1.0% SDS and 15 μl analyzed by SDS-PAGE, stained with Coomassie brilliant blue, fixed and soaked for 15 min in Amplify solution (Amersham). Gels were exposed to Hyperfilm MP (Amersham) at –80°C for three days.

A radioactive band of the appropriate molecular weight was detected in MC58, but not in the Δnmb1870 knockout mutant.

Recombinant *E.coli* grown in the presence of [9,10-^{3}H]-palmitic acid also produce a radioactive band at the expected molecular weight, confirming that *E.coli* recognises the lipoprotein motif and adds a lipid tail to the recombinant protein.

Protein detection

MC58 strain was grown at 37°C with 5% CO_2 in GC medium at stationary phase. Samples were collected during growth (OD_{620nm} 0.05-0.9). MC58Δnmb1870 was grown until OD_{620nm} 0.5. Bacterial cells were collected by centrifugation, washed once with PBS, resuspended in various volumes of PBS in order to standardise the OD values. Culture supernatant was filtered using a 0.2 μm filter and 1 ml precipitated by the addition of 250 μl of 50% trichloroacetic acid (TCA). The sample was incubated on ice for 2 hr, centrifuged for 40 min at 4°C and the pellet washed with 70 % ice cold ethanol, and resuspended in PBS. 3 μl of each sample (corresponding to an OD_{620} 0.03) was then loaded on a 12% polyacrylamide gels and electrotransferred onto nitrocellulose membranes.

Western blot analysis were performed according to standard procedures, using polyclonal antibodies raised against protein expressed in *E.coli*, at a 1:1000 dilution, followed by a 1/2000 dilution of HPR-labeled anti-human IgG (Sigma). Scanning was performed using a LabScan (Pharmacia) and Imagemaster software (Pharmacia).

As shown in Figure 3, a protein of ~29.5 kDa was detected in the total cell extracts of *N.meningitidis*. The amount of the protein in the whole cell lysate approximately doubled during the growth curve, while the optical density of the culture increased from 0.05 to 0.9 OD_{620nm}. A band of the same size was also detected in the culture supernatant. The protein was not detected in the supernatant of the freshly inoculated culture (OD_{620nm} 0.05), and increased approximately four times during the growth from 0.1 to 0.9 OD_{620nm} (Figure 4, left-hand panel). The genuine nature of the expression in the supernatant was confirmed by testing the same samples for membrane blebs and cytoplasmic proteins. As shown in the middle panel of Figure 4, the absence of PorA in the supernatant preparations of PorA rules out a possible contamination with membrane blebs, while the absence in the supernatant of cytoplasmic protein NMB1380 confirmed that the supernatant samples do not result from cell lysis (right-hand panel).

The MC58Δnmb1870 knockout strain shows no protein in either whole cell lysate or culture supernatant (lanes 'KO' in Figures 3 & 4).

NMB1870 was detected by western blotting in outer membrane vesicles, confirming that the protein segregates with the membrane fractions of *N.meningitidis* (Figure 5). However, sera from mice immunised with the OMVs did not recognise recombinant NMB1870 in western blotting, suggesting that the protein is not immunogenic in OMV preparations.

FACS analysis using the anti-NMB1870 antibodies confirmed that the protein is surface-exposed and accessible to antibodies both in encapsulated and non-encapsulated *N.meningitidis* strains (Figure 6). FACS analysis used a FACS-Scan flow cytometer, with antibody binding detected using a secondary antibody anti-mouse (whole molecule) FITC-conjugated (Sigma). The positive FACS control used SEAM3, a mAb specific for the meningococcus B capsular polysaccharide [194]; the negative control consisted of a mouse polyclonal antiserum against the cytoplasmic protein NMB1380 [195].

Western blotting analysis of 43 strains showed that NMB1870 is expressed by all strains tested. As shown in Figure 7, however, the levels of expression varied considerably from strain to strain. The strains tested could be broadly classified as high, intermediate and low expressers:

Strains	**High**	**Intermediate**	**Low**
ET5	9/9	0/9	0/9
Lineage 3	7/9	1/9	1/9
ET37	2/3	1/3	0/3
A4	0/4	2/4	2/4
Other	6/15	7/15	2/15
N. gonorrhoeae	0/1	0/1	1/1
N. cinerea	0/1	0/1	1/1
N. lactamica	1/1	0/1	0/1
Total	**25/43 (58%)**	**11/43 (25.5%)**	**7/43 (16.5%)**

Most of the strains from hypervirulent lineages (ET-5, lineage 3, ET-37) expressed high levels of the protein, with the exception of A4 where two strains expressed intermediate levels and two expressed low levels. Interestingly, the protein was expressed at high level by strains that have been classically

used as OMV vaccine strains. No obvious genetic patterns were found to predict the amount of protein expressed by each strain. Even the presence of the IS element in the promoter region, which was found in 8/70 strains (one from serogroup A, three from lineage 3, and four from those classified as others), did not show any correlation with the expression of the protein.

Scanning of the Western blots showed that the difference in expression between high and intermediate, intermediate and low or high and low could be two-, five- and nine-fold, respectively. There is no immediately-apparent reason for the different expression levels, and analysis of the DNA sequences upstream from the gene did not show any feature that correlates with expression.

Antibody responses

Sera from healthy and convalescent subjects were analysed for anti-NMB1870 antibodies by Western blot. Purified NMB1870 (1 μg/lane) was loaded onto 12.5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The bound protein was detected with 1/200 dilution of sera, followed by a 1/2000 dilution of HPR-labeled anti-human IgG (Sigma). While only 2/10 of sera from healthy people recognised NMB1870, 21/40 convalescent sera recognised the protein, leading to the conclusion that NMB1870 is immunogenic *in vivo* during infection. Antisera from mice immunised with recombinant NMB1870 were therefore investigated further.

To prepare antisera, 20 μg of variant 1, variant 2 and variant 3 NMB1870 recombinant proteins were used to immunise six-week-old CD1 female mice (Charles River). Four to six mice per group were used. The recombinant proteins were given i.p., together with complete Freund's adjuvant (CFA) for the first dose and incomplete Freund's adjuvant (IFA) for the second (day 21) and third (day 35) booster doses. The same immunization schedule were performed using aluminium hydroxide adjuvant (3 mg/ml) instead of Freund's adjuvant. Blood samples for analysis were taken on day 49.

The antisera were tested for their ability to induce complement-mediated killing of capsulated *N.meningitidis* strains, as previously described [3, 196] using pooled baby rabbit serum (CedarLane) used as complement source. Serum from a healthy human adult (with no intrinsic bactericidal activity when tested at a final concentration of 25 or 50%) was also used as complement source. Serum bactericidal titers were defined as the serum dilution resulting in 50% decrease in colony forming units (CFU) per ml after 60 mins. incubation of bacteria with reaction mixture, compared to control CFU per ml at time 0. Typically, bacteria incubated with the negative control antibody in the presence of complement showed a 150 to 200% increase in CFU/ml during the 60 min incubation.

Representative strains from the high, intermediate and low expressors were selected for the assay. The differential expression of the protein on the surface of the selected strains was confirmed by FACS analysis (Figure 8) — MC58, a representative of the high expresser strains was killed with high efficiency by the serum diluted up to 1/64,000; NZ394/98 (originally NZ98/254), a representative of the intermediate expressers was also killed with high efficiency, by the serum diluted up to 1/16,000 and even strain 67/00, a representative of the low expresser strains was killed by the antiserum diluted up to 1/2,048. Control strains, where the nmb1870 gene had been knocked out, was not killed by the same antiserum.

To confirm whether the sera were also able to confer protection *in vivo*, they were tested for ability to induce passive protection in the infant rat model. Five-day-old infant rats were pre-treated i.p. with anti-NMB1870 antisera or with anti-PorA monoclonal antibody at time 0 and challenged two hours later i.p. with 5 x 10^3 CFU/rat of MenC 4243 (OAc-positive) or MenB NZ394/98. Quantitative blood cultures were obtained 24 hours later. Bacterial counts in the blood cultures (CFU/ml, geometric means) were obtained by plating blood on chocolate agar plates. Positive control serum was anti-PorA(P1.2) for MenC and SEAM3 for MenB. Results of the experiments were as follows:

	Challenge Strain 4243		**Challenge Strain NZ394/98**	
Pre-treatment	**Positives**	**CFU/ml x10^3**	**Positives**	**CFU/ml x10^3**
PBS	5/5	450	–	–
Negative control serum	5/5	500	9/14	1260
Positive control serum	1/5	0.003	0/7	<0.001
Anti-NMB1870	0/9	<0.001	0/14	<0.001

Therefore no bacterial colonies were recovered from the blood of the rats passively immunised with anti-NMB1870 serum, while most of the negative control animals were bacteremic.

Bactericidal activity is variant-specific

Each type variant was expressed in *E.coli* as a His-tagged protein and used to immunise mice. The sera were used to test the immunological cross-reactivity between strains of the three variants by FACS and bactericidal assay. As shown in Figure 11, by FACS analysis, all strains were recognised by each serum, although the degree of recognition varied considerably, usually reflecting the amino acid homology between the proteins.

On closer analysis, the anti-variant-1 serum (Figure 11, first row) recognised MC58 strain very well (as expected), to a lower extent the 961-5945 strain (74.1% identity) and, to a lesser extent, the M1239 strain (62.8% identity). A similar trend was found for antisera against variants 2 and 3 (rows 2 and 3 of Figure 11), although with the anti-variant-2 serum the differences were not as striking.

A monoclonal antibody against the capsule recognised all three strains equally well the (row 4), while a serum against the cytoplasmic protein NMB1380 used as negative control did not recognise any (row 5). Similarly, the nmb1870 knock-out mutants were not recognised by any sera (row 6).

The differences in immunorecognition between the variants were more evident by bactericidal assay:

Sera	**MC58** (variant 1)	**961/5945** (variant 2)	**M1239** (variant 3)
Anti-variant 1	64000	256	<4
Anti-variant 2	<4	16000	128
Anti-variant 3	<4	2048	16000

The data show that the serum against each variant was able to induce an efficient complement-mediated killing of the homologous strain (titers ranging between 16,000 and 64,000), while the

activity was low (128-2,048) or absent (<4) against strains of the other variants. As predicted from the close amino acid homology, the cross-bactericidal titers between variants 2 and 3 were higher than the others. When human complement was used, bactericidal titers of 4,096, 256 and 512 were obtained with variants 1, 2 and 3, respectively, using the homologous type strains. No titers were detected against the heterologous strains.

Hybrid and tandem proteins

Hybrid and tandem proteins can be represented by the formula: NH_2-A-[-X-L-]$_n$-B-COOH. Genes encoding various proteins of this type were constructed, where $n=2$, the N-termini of X_1 and X_2 are deleted up to the end of their poly-glycine regions, and -L_2- and -B- are absent (or else B is a poly-histidine tag used for purification). The following table shows the components of these proteins in their mature forms, and gives the SEQ ID NO^S of the full polypeptide and the SEQ ID NO^S and strains for the component sequences A, X_1, L_1 and X_2:

	SEQ ID	A	X_1	L_1	X_2	pI
(1)	79	—	MC58 (SEQ ID 80)	SEQ ID 78	2996 (SEQ ID 81)	6.74
(2)	82	—	MC58 (SEQ ID 80)	SEQ ID 144	2996 (SEQ ID 81)	6.63
(3)	83	—	MC58 (SEQ ID 80)	SEQ ID 78	M1239 (SEQ ID 84)	
(4)	85	—	MC58 (SEQ ID 80)	SEQ ID 144	M1239 (SEQ ID 84)	
(5)	87	SEQ ID 86	2996 (SEQ ID 81)	SEQ ID 78	M1239 (SEQ ID 84)	6.44
(6)	88	SEQ ID 86	2996 (SEQ ID 81)	SEQ ID 144	M1239 (SEQ ID 84)	6.35
(7)	89	SEQ ID 86	M1239 (SEQ ID 84)	SEQ ID 78	2996 (SEQ ID 81)	
(8)	90	SEQ ID 86	M1239 (SEQ ID 84)	SEQ ID 144	2996 (SEQ ID 81)	
(9)	91	Cys	'936' (SEQ ID 76)	SEQ ID 78	2996 (SEQ ID 81)	
(10)	92	Cys	'936' (SEQ ID 76)	SEQ ID 144	2996 (SEQ ID 81)	
(11)	93	Cys	'936' (SEQ ID 76)	SEQ ID 78	M1239 (SEQ ID 84)	
(12)	94	Cys	'936' (SEQ ID 76)	SEQ ID 144	M1239 (SEQ ID 84)	

Of these twelve proteins, therefore, eight are tandem NMB1870 proteins (MW ~ 55kDa) and four are hybrid proteins with '936_{2996}' at the N-terminus (MW ~49kDa). Two linkers were used: (a) SEQ ID NO: 78, which is derived from the gonococcal NMB1870 homolog (SEQ ID NO: 46); and (b) a glycine-rich linker (SEQ ID NO: 144). SEQ ID NO: 78 was also used at the N-terminus of mature proteins, without its two N-terminus *Bam*HI residues (Gly-Ser) *i.e.* SEQ ID NO: 86.

All twelve proteins were soluble when expressed in *E.coli* and, after purification, were used to immunise mice. Serum bactericidal antibody (SBA) responses were assessed against up to four meningococcal strains, ensuring one from each of the three NMB1870 variants 1 to 3 (shown as superscripts). The adjuvant was either CFA (top) or an aluminium hydroxide (bottom):

Protein	SBA			
	2996 (2)	MC58 (1)	M1239 (3)	961/5945 (2)
(1)	4096 1024	262144 32768	2048 128	32768 2048
(2)	8192 1024	262144 16384	2048 512	32768 1024
(3)	— —	131072 32768	32768 4096	4096 1024
(4)	— —	262144 32768	32768 1024	8192 512
(5)	512 1024	<4 16	4096 4096	32768 8192
(6)	4096 2048	<4 16	32768 4096	32768 16384
(7)	— —	4 16	4096 4096	32768 8192
(8)	2048 1024	32 32	32768 8192	32768 16384
(9)	2048 4096	<4 <4	<4 512	32768 16384
(10)	4096 512	<4 <4	256 <4	131072 2048
(11)	256 4	<4 4	>32768 4096	2048 128
(12)	2048 16	<4 256	>32768 4096	4096 256

These results clearly show the variant-specific nature of the immune reactions. For example, proteins (1) and (2) include sequences from NMB1870 variants 1 and 2, and the best SBA results are seen against these two variants. Similarly, the best results are seen against variants 1 and 3 when using proteins (3) and (4). Good activity is seen using NMB1870 from variants 2 and 3, in either order from N-terminus to C-terminus, using proteins (5) to (8), with little activity against variant 1. The variant-specific nature of the NMB1870 response is also apparent when using the hybrid proteins, with some anti-2996 activity being provided by the '936' moiety.

The following oligonucleotide primers were using during the construction of the 12 proteins:

Protein	Primer SEQ ID NO^S (Fwd & Rev)	Restriction sites
(1)	95 & 96	*Bam*HI & *Xho*I
(2)	97 & 98	*Bam*HI & *Xho*I
(3)	99 & 100	*Bam*HI & *Hin*dIII
(4)	101 & 102	*Bam*HI & *Hin*dIII
(5)	103 & 104	*Bam*HI & *Hin*dIII
(6)	105 & 106	*Bam*HI & *Hin*dIII
(7)	107 & 108	*Bam*HI & *Xho*I
(8)	109 & 110	*Bam*HI & *Xho*I
(9)	111 & 112	*Bam*HI & *Xho*I
(10)	113 & 114	*Bam*HI & *Xho*I
(11)	115 & 116	*Bam*HI & *Hin*dIII
(12)	117 & 118	*Bam*HI & *Hin*dIII
$NMB1870_{M1239}$	119 & 120	*Nde*I & *Bam*HI
$NMB1870_{2996}$	121 & 122	*Nde*I & *Bam*HI

Triple tandem protein

A "triple tandem" protein, where *n=3*, was constructed based on strains (1) MC58, (2) 2996 and (3) m1239. The 757mer triple tandem protein NH_2-A-X_1-L_1-X_2-L_2-X_3-L_3-B-COOH has amino acid sequence SEQ ID NO: 142:

Moiety	A	X_1	L_1	X_2	L_2	X_3	L_3	B
Detail	–	$NMB1870_{MC58}$	Gly-rich linker	$NMB1870_{2996}$	Gly-rich linker	$NMB1870_{m1239}$	–	–
SEQ ID	–	80	144	81	144	84	–	–
Variant	–	1	–	2	–	3	–	–

X_2 and X_3 both lack the N-terminus up to their poly-glycine regions (*i.e.* they are ΔG sequences).

Bactericidal SBA titres

Mice were immunised with nine different proteins and the bactericidal activity of the resulting sera were tested against different strains of meningococcus, including both strains which match those from which the immunising proteins were derived and strains which are different from the immunising proteins. The nine proteins were:

(A), (B) & (C) Hybrid proteins of 936 and NMB1870

(A) $NMB1870_{MC58}$ = variant 1 [12]

(B) $NMB1870_{2996}$ = variant 2 *e.g.* SEQ ID NO^S: 91 & 92

(C) $NMB1870_{M1239}$ = variant 3 *e.g.* SEQ ID NO^S: 91 & 92

(D), (E) & (F) NMB1870 from single strains

(D) $NMB1870_{MC58}$ = variant 1 *e.g.* SEQ ID NO: 80

(E) $NMB1870_{2996}$ = variant 2 *e.g.* SEQ ID NO: 81

(F) $NMB1870_{M1239}$ = variant 3 *e.g.* SEQ ID NO: 84

(G), (H) & (I) NMB1870 tandem proteins

(G) $NMB1870_{MC58}$—$NMB1870_{2996}$ = variants 1 & 2 *e.g.* SEQ ID NO^S: 79 & 82

(H) $NMB1870_{2996}$—$NMB1870_{M1239}$ = variants 2 & 3 *e.g.* SEQ ID NO^S: 87 & 88

(I) $NMB1870_{MC58}$—$NMB1870_{M1239}$ = variants 1 & 3 *e.g.* SEQ ID NO^S: 83 & 85

Bactericidal responses were measured against up to 20 strains which possess variant 1 of NMB1870, against up to 22 strains with variant 2 of NMB1870 and against up to 5 strains with variant 3.

The bactericidal efficacy of sera raised against proteins (A) to (C) matched the genotype of the test strains *e.g.* using CFA as adjuvant for the immunisations, the SBA titres against strain MC58 (variant 1) were: <u>(A) 262144</u>; (B) <4; (C) <4. Similarly, when sera were tested against strain 961-5945 (variant 2) the SBA were: (A) 256; <u>(B) 32768</u>; (C) 4096. Finally, against strain M1239 (variant 3) titres were: (A) <4; (B) 512; <u>(C) 32768</u>.

Using CFA or aluminium hydroxide as adjuvant, protein (A) gave SBA titres of ≥512 against the following strains: M01-240185, M2197, LPN17592, M6190 (all ET37); MC58, BZ83, CU385, N44/89, 44/76, M2934, M4215 (all ET5); BZ133; M1390, ISS1026, ISS1106, ISS1102 (lin. 3);

F6124 (sIII); and M2937 (other). These strains cover serogroups A, B, C and W135; no serogroup Y strains were tested.

Using CFA or aluminium hydroxide as adjuvant, protein (B) gave SBA titres ≥512 against strains: 2996, 961-5945, 96217 (cluster A4); M01-240013, C11, NGH38, M3279, M4287, BZ232 (other). These strains cover serogroups B and C; no serogroup A, W135 or Y strains were tested.

Using CFA or aluminium hydroxide as adjuvant, protein (C) gave SBA titres ≥512 against strains: M01-0240364, NGP165 (ET37); M1239 (lin. 3); M01-240355, M3369 (other). These strains are in serogroup B, and no serogroup A, C, W135 or Y strains were tested.

The SBA patterns seen with proteins (A) to (C) were also seen with proteins (D) to (F). Against strain MC58, serum obtained using protein (D) and aluminium hydroxide adjuvant gave a SBA titre of 16384, whereas sera obtained using protein (E) or (F) and the same adjuvant gave SBA titres <4. Against strain 961-5945, protein (D) and (F) sera gave lower titres than those obtained using (E). Against, strain M1239, SBA titres were: (D) <4; (E) 128; (F) 16384.

With tandem proteins, SBA efficacy was broadened. Sera obtained using protein (G) were bactericidal against strain MC58 and 961-5945, as well as other strains which possess variant 1 or variant 2 of NMB1870. Sera raised against protein (H) gave low titres against strains which posssess variant 1 of NMB1870, but high titres against other strains *e.g.* 16384 against strain 961-5945 (variant 2) and 32768 against strain M3369 (variant 3).

Sera obtained by immunisation with CFA-adjuvanted protein (H) gave SBA titres ≥512 against: LNP17094, 96217, 961-5945, 2996, 5/99 (cluster A4); C4678, M01-0240364, NGP165 (ET37); M1239 (lin. 3); M2552, BZ232, M3279, M4287, 1000, NGH38, C11, M01-240013, M01-240355, M3369 (other). These strains cover serogroups B and C; activity against serogroups A, W135 or Y strains was not tested with protein (H).

Sera obtained by immunisation with CFA-adjuvanted protein (I) gave SBA titres ≥512 against: M01-0240364, 14784, M6190, MC58, LPN17592, M2197 (ET37); 44/76 (ET5); M1239, ISS1102, ISS1106, ISS1026, 394/98 (lin. 3); M2937 (other). These strains cover serogroups B, C and W135; activity against serogroups A or Y strains was not tested with protein (I).

After immunisation with proteins containing variant 1 of NMB1870, sera tested against up to 20 strains which have a NMB1870 in variant 1 gave SBA titres as follows:

Protein	(A)	(A)	(D)	(D)	(G)	(G)	(I)	(I)
Adjuvant	CFA	Alum	CFA	Alum	CFA	Alum	CFA	Alum
N° strains tested	18	20	20	20	13	13	11	11
SBA <128	1	7	4	3	0	5	0	2
SBA 128-512	2	4	0	4	0	3	0	3
SBA >512	15	9	16	13	13	5	11	6

After immunisation with proteins containing variant 2 of NMB1870, sera tested against up to 22 strains which have a NMB1870 in variant 2 gave SBA titres as follows:

Protein	(B)	(B)	(E)	(G)	(G)	(H)	(H)	*(I)*	*(I)*
Adjuvant	CFA	Alum	Alum	CFA	Alum	CFA	Alum	*CFA*	*Alum*
N° strains tested	16	19	22	16	15	22	22	*7*	*6*
SBA <128	6	14	13	6	10	7	8	*3*	*5*
SBA 128-512	0	2	7	3	3	1	6	*0*	*1*
SBA >512	10	3	2	7	2	14	8	*4*	*0*

After immunisation with proteins containing variant 3 of NMB1870, sera tested against up to 5 strains which have a NMB1870 in variant 3 gave SBA titres as follows:

Protein	(C)	(C)	(F)	*(G)*	*(G)*	(H)	(H)	(I)	(I)
Adjuvant	CFA	Alum	Alum	*CFA*	*Alum*	CFA	Alum	CFA	Alum
N° strains tested	5	5	5	*5*	*5*	5	5	3	3
SBA <128	0	1	1	*1*	*1*	0	0	1	1
SBA 128-512	0	0	0	*0*	*2*	0	0	0	1
SBA >512	5	4	4	*4*	*2*	5	5	2	1

Conclusions

At first, NMB1870 appears not to be a useful antigen for broad immunisation — its expression levels vary between strains, there is significant sequence variability, and there is no cross-protection between the different variants. However, it has been shown that even those strains which express very low levels of this antigen are susceptible to anti-NMB1870 sera. Furthermore, sequence diversity is limited to three variant forms such that broad immunity can be achieved without the need for a large number of antigens. In addition, it seems that these three proteins may offer immunity against more than just serogroup B meningococcus.

The different variants of NMB1870 can be expressed together as fusion proteins in order to give single polypeptide chains which are active against more than one variant.

NMB1870 is immunogenic during infection, is able to induce bactericidal antibodies, and protects infant rats from bacterial challenge.

Further experimental information on NMB1870 can be found in reference 197.

It will be understood that the invention is described above by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO:	Description
1-23	23 different NMB1870 sequences, full-length
24-45	22 different NMB1870 sequences, with N-terminus cysteines
46	N-terminal amino acid sequence used for expression
47-66	PCR primers
67-69	Partial sequences from Figure 1
70-73 & 86	Sequence motifs for retention or omission from proteins of the invention
74-75	Fur boxes
76	'936' from MC58, with leader peptide processed
77	Example of a 936_{MC58}—ΔG-NMB1870$_{M1239}$ hybrid
78	Gonococcus-derived sequence used for chimeric expression
79, 82, 83, 85, 87, 88, 89, 90	Tandem NMB1870 proteins
80, 81, 84	Truncated NMB1870 sequences
91-94	Hybrid proteins of '936' and NMB1870
95-122	PCR primers
123-141	Full-length NMB1870 sequences
142	Triple tandem NMB1870 sequence
143	Haji NadA sequence
144	Glycine linker

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CLAIMS

1. A composition comprising at least two of the following antigens: (a) a first protein, comprising an amino acid sequence having at least 85% sequence identity to SEQ ID NO: 24 and/or comprising an amino acid sequence consisting of a fragment of at least 7 contiguous amino acids from SEQ ID NO: 24; (b) a second protein, comprising an amino acid sequence having at least 85% sequence identity to SEQ ID NO: 33 and/or comprising an amino acid sequence consisting of a fragment of at least 7 contiguous amino acids from SEQ ID NO: 33; and (c) a third protein, comprising an amino acid sequence having at least 85% sequence identity to SEQ ID NO: 41 and/or comprising an amino acid sequence consisting of a fragment of at least 7 contiguous amino acids from SEQ ID NO: 41.

2. The composition of claim 1, wherein: protein (a) has less than 70% sequence identity to protein (b); protein (a) has less than 70% sequence identity to protein (c); and protein (b) has less than 70% sequence identity to protein (c).

3. The composition of any preceding claim, wherein the composition can elicit a bactericidal response effective against each of serogroup B *N.meningitidis* strains MC58, 961-5945 and M1239.

4. The composition of any preceding claim, wherein the composition can elicit an antibody response which is bactericidal against *N.meningitidis* strains in at least 2 of hypervirulent lineages ET-37, ET-5, cluster A4, lineage 3, subgroup I, subgroup III, and subgroup IV-1.

5. The composition of any preceding claim, wherein one or more of the proteins is a lipoprotein.

6. The composition of any preceding claim, wherein at least one of the proteins does not include the amino acid sequence `TRSKP` (SEQ ID NO: 70) or `TRSKPV` (SEQ ID NO: 71) within 10 amino acids of its N-terminus.

7. The composition of any preceding claim, wherein at least one of the proteins does not include the amino acid sequence `PSEPPFG` (SEQ ID NO: 72) within 10 amino acids of its N-terminus.

8. The composition of any preceding claim, wherein at least one of the proteins includes the amino acid sequence `GGGG` (SEQ ID NO: 73).

9. The composition of any preceding claim, wherein at least one of the proteins is used in the form of a fusion protein.

10. The composition of claim 9, wherein the fusion protein includes SEQ ID NO: 46 and/or the *H.influenzae* P4 lipoprotein leader sequence.

11. The composition of any preceding claim, comprising one or more proteins comprising an amino acid sequence selected from SEQ ID NO^{S}: 1-45, 77, 79-85, 87-94, and 123-142.

12. A composition comprising a hybrid protein of formula NH_2-A-[-X-L-]$_n$-B-COOH, wherein: *n* is 2 or more; L is an optional linker amino acid sequence; A is an optional N-terminal amino acid sequence; B is an optional C-terminal amino acid sequence; and the X moieties include at least two of: (a) a first protein, comprising an amino acid sequence having at least 85% sequence identity to SEQ ID NO: 24 and/or comprising an amino acid sequence consisting of a fragment of at least 7 contiguous amino acids from SEQ ID NO: 24; (b) a second protein, comprising an amino acid sequence having at least 85% sequence identity to SEQ ID NO: 33 and/or comprising an amino acid sequence consisting of a fragment of at least 7 contiguous amino acids from SEQ ID NO: 33; and (c) a third protein, comprising an amino acid sequence having at least 85% sequence identity to SEQ ID NO: 41 and/or comprising an amino acid sequence consisting of a fragment of at least 7 contiguous amino acids from SEQ ID NO: 41.

13. The composition of claim 12, wherein the hybrid protein comprises one of the following amino acid sequences: SEQ ID NO^{S}: 79, 82, 83, 85, 87, 88, 89, 90 and 142.

14. The composition of any preceding claim, including fewer than 15 antigens.

15. The composition of any preceding claim, comprising a Neisserial antigen other than a NMB1870 protein.

16. The composition of any preceding claim, comprising a vesicle prepared from *N.meningitidis*.

17. The composition of any preceding claim, comprising a saccharide antigen from *N.meningitidis* serogroup A, C, W135 and/or Y.

18. The composition of claim 17, comprising a saccharide antigen from *N.meningitidis* serogroups A, C, W135 and Y.

19. The composition of any preceding claim, comprising a saccharide antigen from *Haemophilus influenzae* type B.

20. The composition of claim 17, claim 18 or claim 19, wherein the saccharide antigen(s) is/are conjugated to one or more carrier proteins.

21. The composition of claim 17, claim 18, claim 19 or claim 20, wherein the saccharide antigen(s) are oligosaccharides.

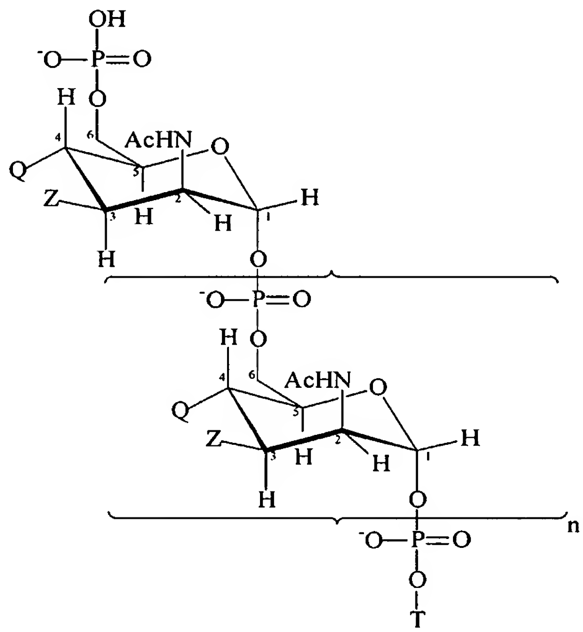
22. The composition of any preceding claim, comprising an antigen from *Streptococcus pneumoniae*.

23. The composition of any one of claims 17 to 22, wherein the serogroup A saccharide antigen is modified saccharide in which one or more of the hydroxyl groups on the native saccharide has/have been replaced by a blocking group.

24. The composition of any one of claims 17 to 22, wherein, where a serogroup A saccharide antigen contains *n* monosaccharide units, at least 50% of the monosaccharide units do not have –OH groups at both of positions 3 and 4.

25. The composition of any one of claims 17 to 22, wherein the serogroup A saccharide comprises monosaccharide units, wherein at least 1 of the monosaccharide units does not have –OH at the 3 position and do not have –OH at the 4 position.

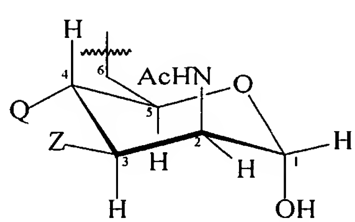
26. The composition of any one of claims 17 to 25, wherein the serogroup A saccharides have the formula:



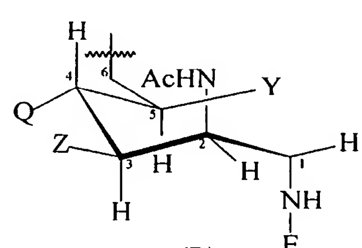
, wherein

n is an integer from 1 to 100 (preferably an integer from 15 to 25);

T is of the formula (A) or (B):



(A)



(B)

each Z group is independently selected from OH or a blocking group; and

each Q group is independently selected from OH or a blocking group;

Y is selected from OH or a blocking group;

E is H or a nitrogen protecting group;

and wherein more than 7% of the Q groups are blocking groups.

27. The composition of claim 20, wherein the carrier protein is a diphtheria toxoid, a tetanus toxoid, CRM_{197}, a *N.meningitidis* outer membrane protein, protein D from *H.influenzae*, or pneumococcal surface protein PspA.

28. The composition of any preceding claim, comprising: (i) at least two of said antigens (a), (b) and/or (c); (ii) a saccharide antigen from each of *N.meningitidis* serogroups A, C, W135 and Y; (iii) a saccharide antigen from *Haemophilus influenzae* type B; and (iv) an antigen from *Streptococcus pneumoniae.*

29. The composition of any preceding claim, for use as a medicament.

30. A method for raising an antibody response in a mammal, comprising administering the composition of any preceding claim to the mammal.

31. The method of claim 30, wherein the method protects a mammal against a Neisserial infection.

32. The use of at least two of antigens (a), (b) and (c) as defined in claim 1, in the manufacture of a medicament for preventing Neisserial infection in a mammal.

33. Nucleic acid encoding the protein of claim 12 or claim 13.

FIGURE 1

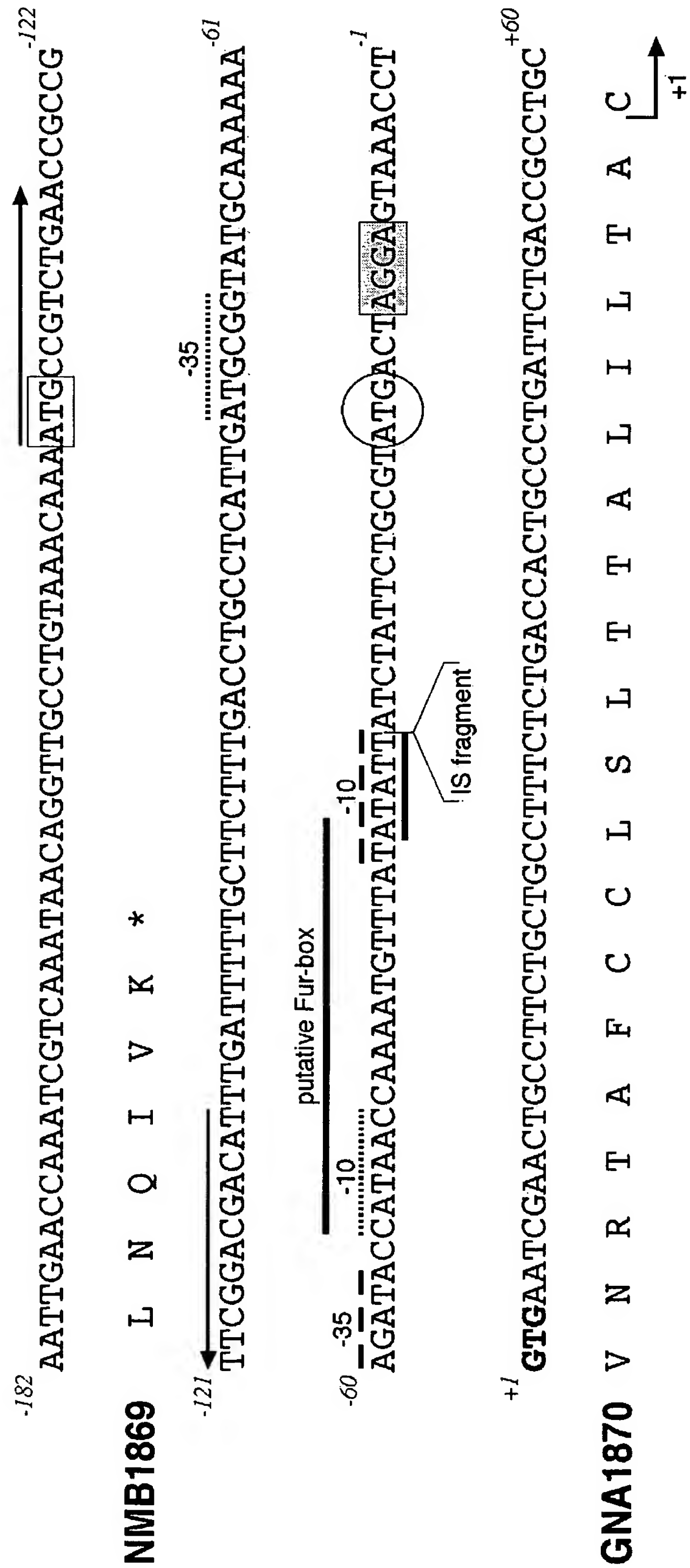

-182 AATTGAACCAAATCGTCAAATAACAGGTTGCCTGTAAACAAAATGCCGTCTGAACCGCG -122
NMB1869 L N Q I V K *
-121 TTCGGACGACATTTGATTTTTGCTTCTTTGACCTGCCTCATTGATGCGGTATGCAAAAA -61
-35
putative Fur-box
-35 -10 -10
-60 AGATACCATAACCAAAATGTTATATATTATCTATTCTGCGTATGACTAGGAGTAAACCT -1
IS fragment
+1 GTGAATCGAACTGCCTTCTGCTGCCTTTCTCTGACCACTGCCCTGATTCTGACCGCCTGC +60
GNA1870 V N R T A F C C L S L T T A L I L L T A C
+1

FIGURE 2

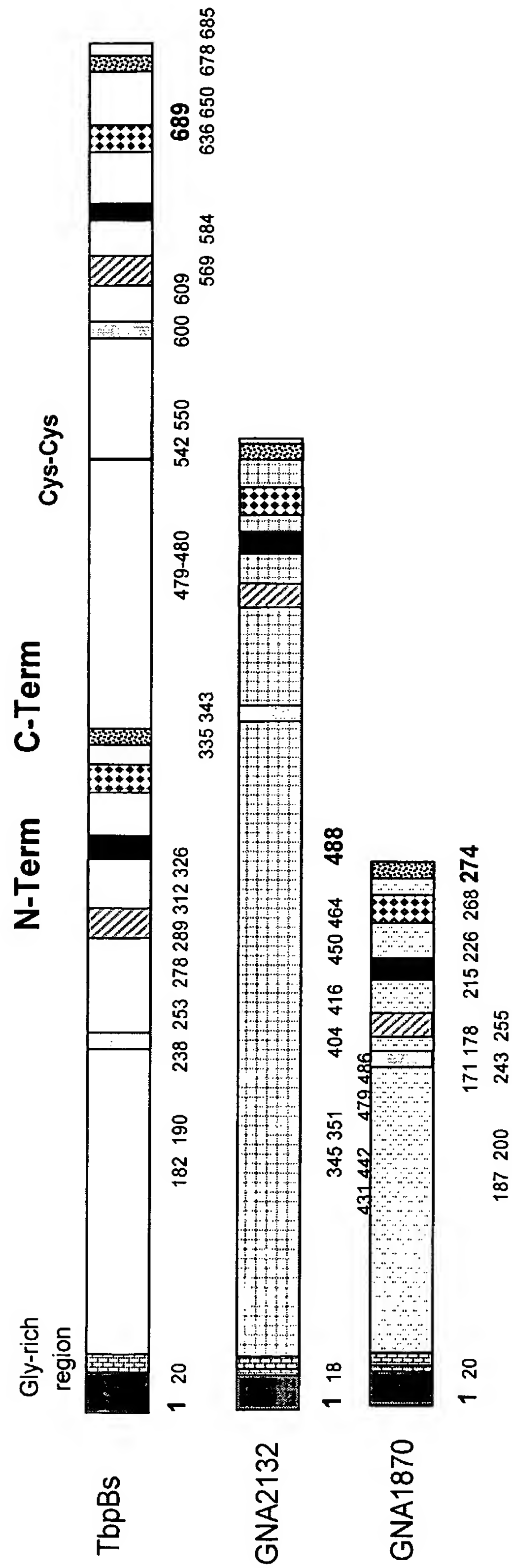

TbpBs
GNA2132
GNA1870
Gly-rich region
N-Term
C-Term
Cys-Cys
689
488
274

FIGURE 3

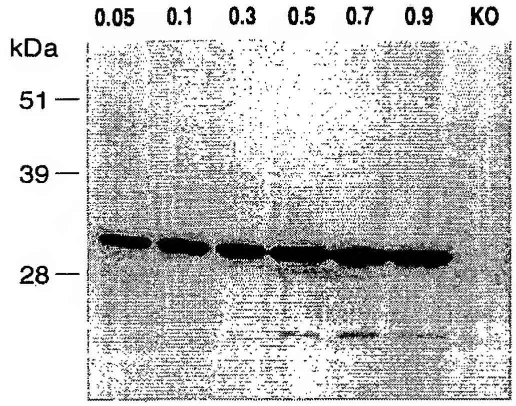


FIGURE 4

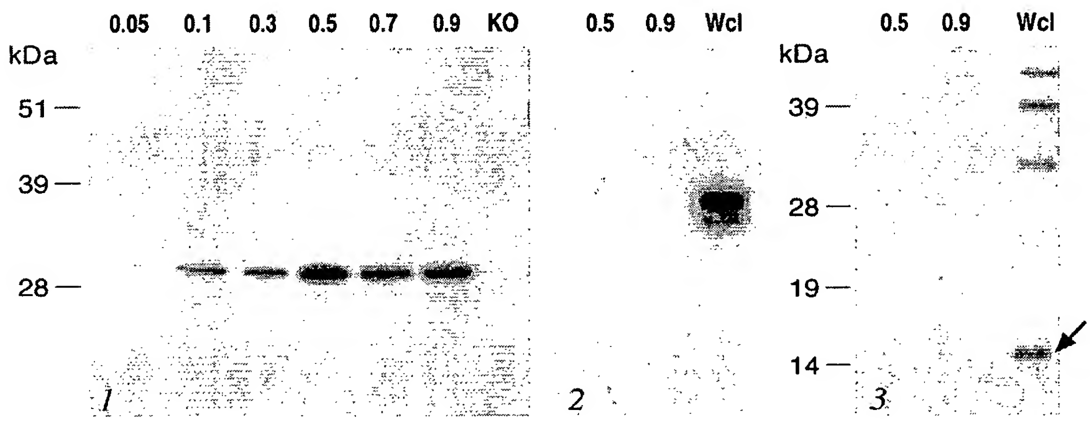


FIGURE 5

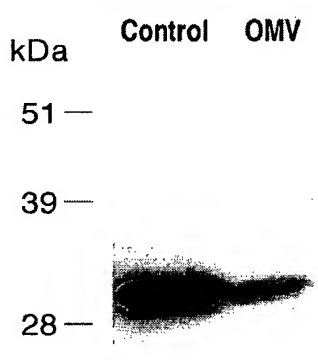


FIGURE 6

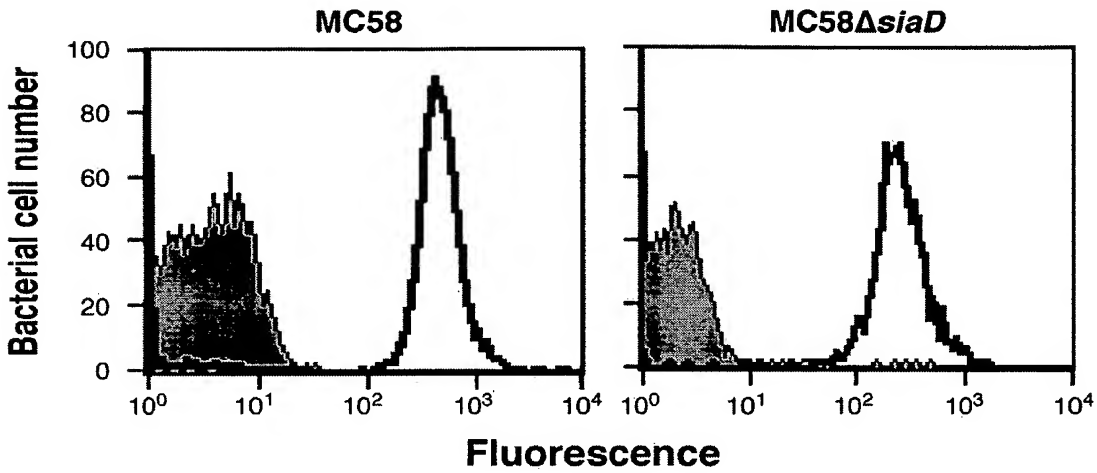


FIGURE 7

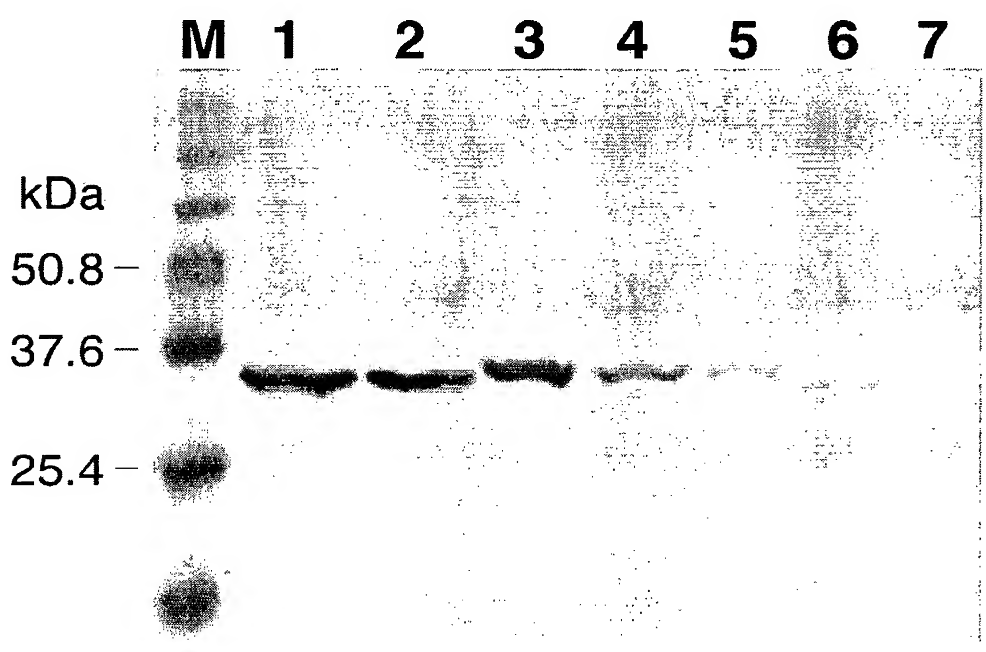


FIGURE 8

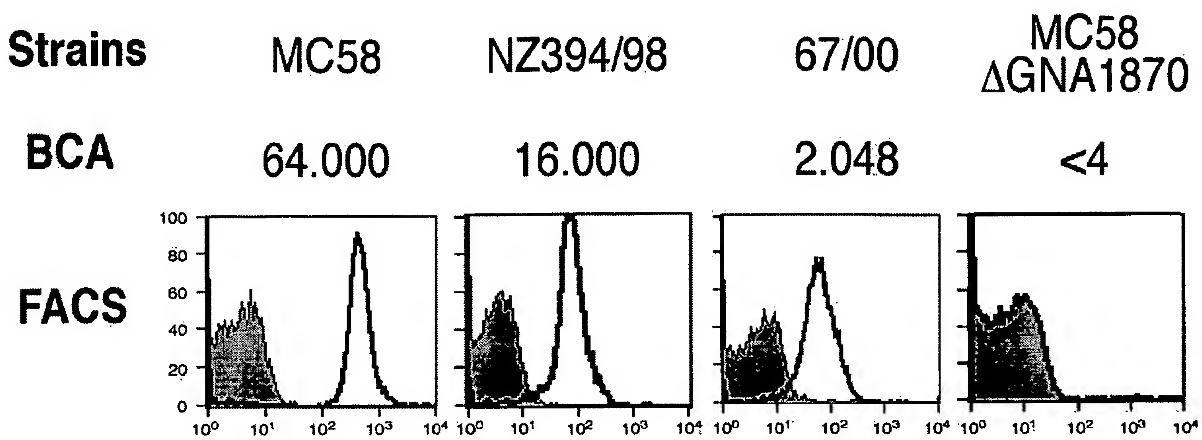


FIGURE 9

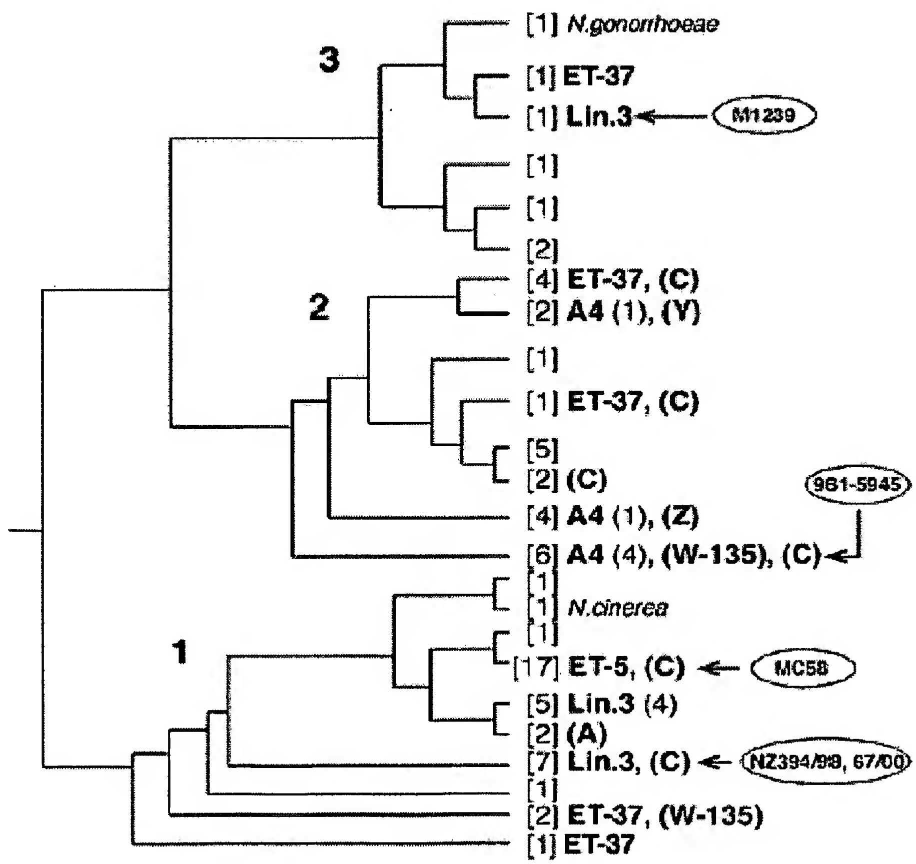


FIGURE 10

```
                                  1
type 1  -19:VNRTAFCCLSLTTALILTACS.....SGGGGVAADIGAGLADALTAPLDH: 26
type 2  -19:VNRTAFCCLSLTAALILTACS.....SGGGGVAADIGAGLADALTAPLDH: 26
type 3  -19:VNRTAFCCLSLTTALILTACSSGGGGSGGGGVAADIGTGLADALTAPLDH: 31

type 1   27:KDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGD...SLNTGKLKNDKV: 73
type 2   27:KDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGD...SLNTGKLKNDKV: 73
type 3   32:KDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKI: 81

type 1   74:SRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTAFQTEQIQDSEHSGKM:123
type 2   74:SRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSL:123
type 3   82:SRFDFVQKIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKTDSL:131

type 1  124:VAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFA:173
type 2  124:INQRSFLVSGLGGEHTAFNQLP.DGKAEYHGKAFSSDDAGGKLTYTIDFA:172
type 3  132:INQRSFLVSGLGGEHTAFNQLP.GGKAEYHGKAFSSDDPNGRLHYSIDFT:180

type 1  174:AKQGNGKIEHLKSPELNVDLAAADIKPDGKRHAVISGSVLYNQAEKGSYS:223
type 2  173:AKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYH:222
type 3  181:KKQGYGRIEHLKTLEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYH:230

type 1  224:LGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQ:255
type 2  223:LALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ:254
type 3  231:LALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ:262
```

FIGURE 11

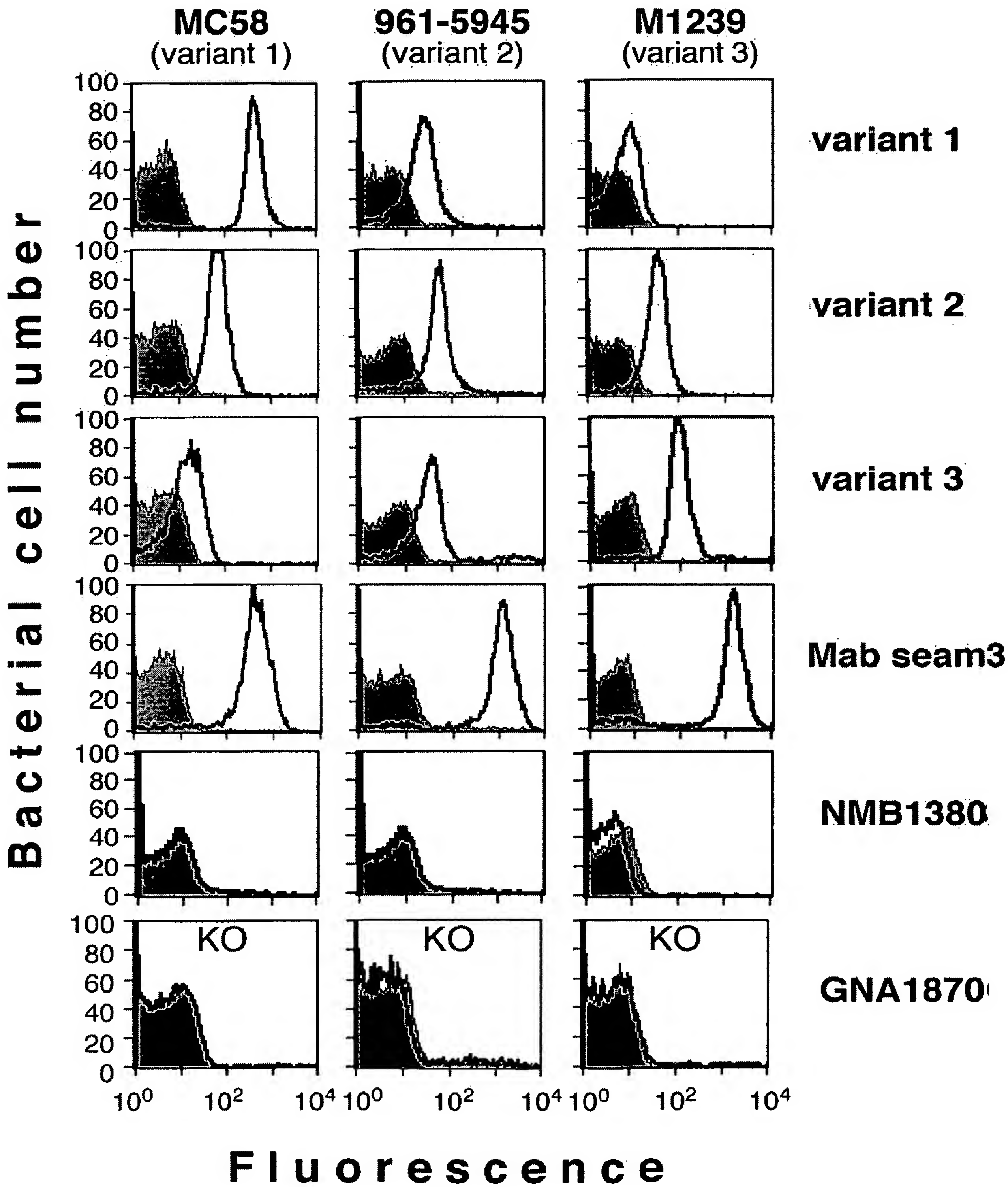

MC58
(variant 1)
961-5945
(variant 2)
M1239
(variant 3)
variant 1
variant 2
variant 3
Mab seam3
NMB1380
GNA1870
KO
KO
KO
Bacterial cell number
100
80
60
40
20
0
10^0
10^2
10^4
Fluorescence

FIGURE 12


ST44cpx (26)
ST44cpx
ST1403
ST11cpx (3)
ST11cpx
ST60 (2)
ST913
ST60
ST178
ST23cpx
ST11cpx
ST8cpx
ST11cpx
ST35cpx
ST11cpx
ST32cpx (33)
ST44cpx (9)
ST2272
subgroup III
subgroup I
ST428
ST1194
subgroup I

FIGURE 13

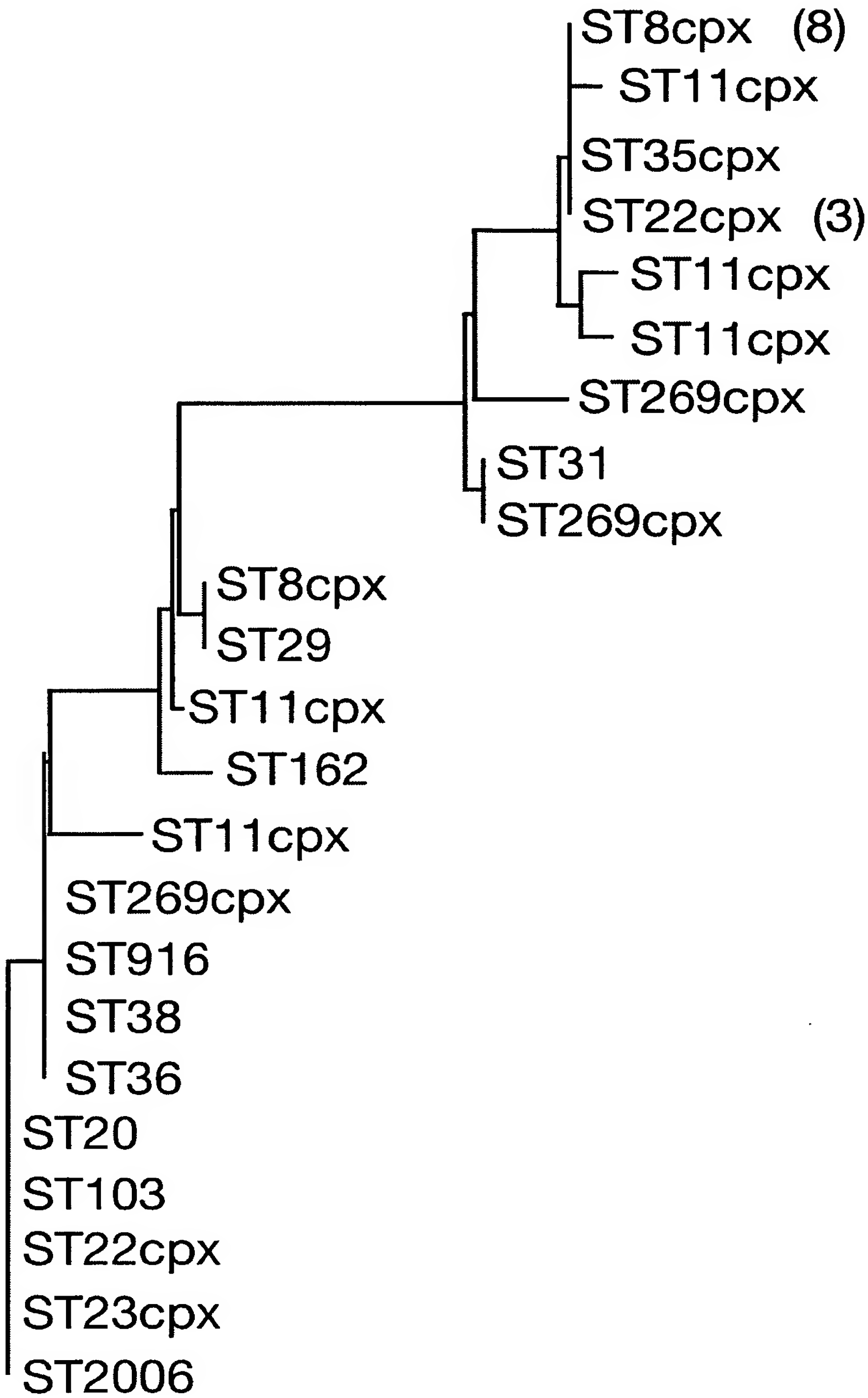
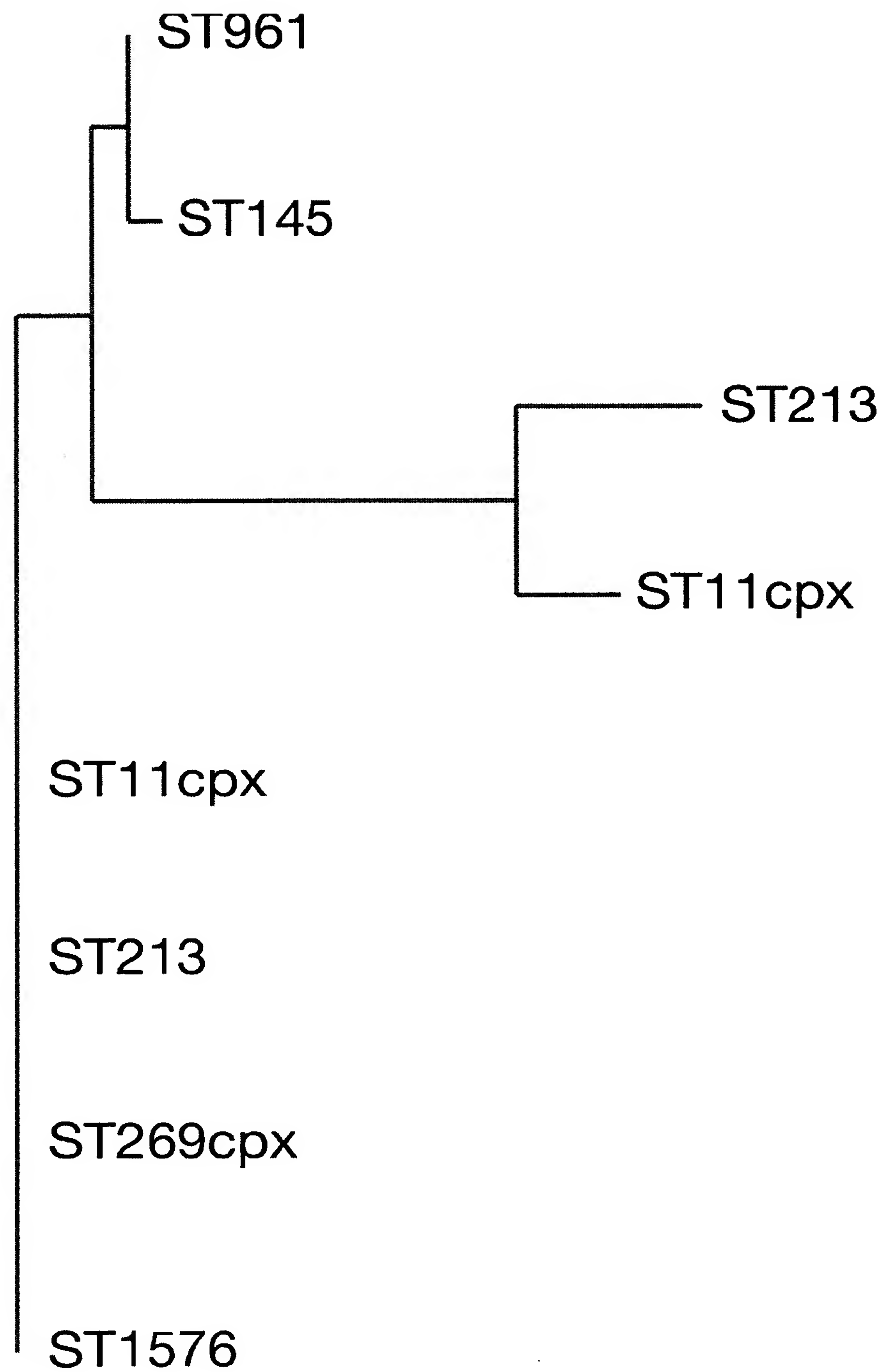

ST8cpx (8)
ST11cpx
ST35cpx
ST22cpx (3)
ST11cpx
ST11cpx
ST269cpx
ST31
ST269cpx
ST8cpx
ST29
ST11cpx
ST162
ST11cpx
ST269cpx
ST916
ST38
ST36
ST20
ST103
ST22cpx
ST23cpx
ST2006

FIGURE 14



SEQUENCE LISTING

SEQ ID NO: 1 – strain MC58 [WO99/57280]

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FIRQIEVDGQLITLESGEFQVYKQSHSALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFA
AKQGNGKIEHLKSPELNVDLAAADIKPDGKRHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQ

SEQ ID NO: 2 – strain gb185

MNRTAFCCLSLTTALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLMLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGKLITLESGEFQVYKQSHSALTALQTEQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKGGSATYRGTAFGSDDAGGKLTYTIDFA
AKQGHGKIEHLKSPELNVELATAYIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 3 – strain m4030

MNRTAFCCFSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKGGSATYRGTAFGSDDAGGKLTYTIDFA
AKQGHGKIEHLKSPELNVELATAYIKPDEKRHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 4 – strain iss1001

MNRTAFCCFSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQEQDPEHSGKMVAKRRFKIGDIAGEHTSFDKLPKDVMATYRGTAFGSDDAGGKLTYTIDFA
AKQGHGKIEHLKSPELNVELATAYIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 5 – strain lnp17592

MNRTTFFCLSLTAALILTACSSGGGGSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDK
VSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKGGSATYRGTAFGSDDAGGKLTY
TIDFAVKQGHGKIEHLKSPELNVDLAAAYIKPDKKRHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 6 – strain f6124

MNRTAFCCLSLTAALILTACSSGGGGVAADIGAVLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDASGKLTYTIDFA
AKQGHGKIEHLKSPELNVDLAASDIKPDKKRHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGSAEVETANGIRHIGLAAKQ

SEQ ID NO: 7 – strain m198172

MNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDASGKLTYTIDFA
AKQGHGKIEHLKSPELNVDLAASDIKPDKKRHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGSAEVETANGIRHIGLAAKQ

SEQ ID NO: 8 – strain m2197

MNRTAFCCLSLTTALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLMLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
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AKQGHGKIEHLKSPELNVDLAAAYIKPDEKHHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQ

SEQ ID NO: 9 – strain m2937

MNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLRSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQEQDLEHSGKMVAKRRFRIGDIAGEHTSFDKLREGGRATYRGTAFGSDDAGGKLTYTIDFA
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SEQ ID NO: 10 – strain 961-5945

MNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
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KQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 11 – strain gb013

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KQGHGKIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 12 – strain 860800
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KQGYGRIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 13 – strain 95n477
MNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPSGKAEYHGKAFSSDDPNGRLHYSIDFTK
KQGYGRIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 14 – strain m2671
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KQGYGRIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 15 – strain 1000
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FIRQIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDPNGRLHYSIDFTK
KQGYGRIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 16 – strain m3279
MNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDPNGRLHYSIDFTK
KQGYGRIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 17 – strain 193-4286
MNRTAFCCLSLTTALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLMLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDPNGRLHYSIDFTK
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SEQ ID NO: 18 – strain m1239
MNRTAFCCLSLTTALILTACSSGGGGSGGGGVAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLK
NDKISRFDFVQKIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRL
HYSIDFTKKQGYGRIEHLKTLEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 19 – strain 16889
MNRTAFCCLFLTTALILTACSSGGGGSGGGGVAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLK
NDKISRFDFVQKIEVDGQTITLASGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDAGGKL
TYTIDFAAKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEISIAGKQ

SEQ ID NO: 20 – strain gb355
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NDKISRFDFVQKIEVDGQTITLASGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDAGGKL
TYTIDFAAKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 21 – strain m3813
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KISRFDFVQKIEVDGQTITLASGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDAGGKLTY
TIDFAAKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 22 – strain ngp165
MNRTTFCCLSLTTALILTACSSGGGGSGGGGVAADIGAGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGGKDNSLNTGKLK
NDKISRFDFVQKIEVDGQTITLASGEFQIYKQDHSAVVALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRL
HYTIDFTNKQGYGRIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 23 – strain fa1090
MNRTTFCCLSLTAGPDSDRLQQRRGGGGGVAADIGTGLADALTAPLDHKDKGLKSLTLEASIPQNGTLTLSAQGAEKTFKAGGKDNSLNTGKLKND
KISRFDFVQKIEVDGQTITLASGEFQIYKQDHSAVVALRIEKINNPDKIDSLINQRSFLVSDLGGEHTAFNQLPDGKAEYHGKAFSSDDADGKLTY
TIDFAAKQGHGKIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYRLALFGDRAQEIAGSATVKIGEKVHEIGIADKQ

SEQ ID NO: 24 – strain MC58

CSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEF
QVYKQSHSALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFAAKQGNGKIEHLKSPELNVD
LAAADIKPDGKRHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQ

SEQ ID NO: 25 – strain gb185

CSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLMLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGKLITLESGEF
QVYKQSHSALTALQTEQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKGGSATYRGTAFGSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVE
LATAYIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 26 – strain m4030

CSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEF
QVYKQSHSALTALQTEQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKGGSATYRGTAFGSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVE
LATAYIKPDEKRHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 27 – strain iss1001

CSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEF
QVYKQSHSALTALQTEQEQDPEHSGKMVAKRRFKIGDIAGEHTSFDKLPKDVMATYRGTAFGSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVE
LATAYIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 28 – strain lnp17592

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ESGEFQVYKQSHSALTALQTEQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKGGSATYRGTAFGSDDAGGKLTYTIDFAVKQGHGKIEHLKSP
ELNVDLAAAYIKPDKKRHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 29 – strain f6124

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QVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDASGKLTYTIDFAAKQGHGKIEHLKSPELNVD
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SEQ ID NO: 30 – strain m198172

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QVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDASGKLTYTIDFAAKQGHGKIEHLKSPELNVD
LAASDIKPDKKRHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGSAEVETANGIRHIGLAAKQ

SEQ ID NO: 31 – strain m2197

CSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLMLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEF
QVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLIYTIDFAAKQGHGKIEHLKSPELNVD
LAAAYIKPDEKHHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQ

SEQ ID NO: 32 – strain m2937

CSSGGGGVAADIGAGLADALTAPLDHKDKGLRSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEF
QVYKQSHSALTALQTEQEQDLEHSGKMVAKRRFRIGDIAGEHTSFDKLREGGRATYRGTAFGSDDAGGKLTYTIDFAAKQGYGKIEHLKSPELNVD
LAAADIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGEAQEVAGSAEVKTANGIHHIGLAAKQ

SEQ ID NO: 33 – strain 961-5945

CSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEF
QIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVEL
AAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 34 – strain gb013

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ASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 35 – strain 860800

CSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEF
QIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPSGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTPEQNVEL
ASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 36 – strain 95n477
CSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEF
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SEQ ID NO: 37 – strain m2671
CSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEF
QIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTPEQNVEL
ASAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 38 – strain 1000
CSSGGGGVAADIGAGLADALTTPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQTITLASGEF
QIYKQNHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTPEQNVEL
ASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 39 – strain m3279
CSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQTITLASGEF
QIYKQNHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTPEQNVEL
ASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 40 – strain l93-4286
CSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLMLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQTITLASGEF
QIYKQNHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTPEQNVEL
ASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 41 – strain m1239
CSSGGGGSGGGGVAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQT
ITLASGEFQIYKQNHSAVVALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLK
TLEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 42 – strain 16889
CSSGGGGSGGGGVAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQT
ITLASGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLK
TPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEISIAGKQ

SEQ ID NO: 43 – strain gb355
CSSGGGGSGSGGVAADIGTGLADALTTPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQT
ITLASGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLK
TPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 44 – strain m3813
CSSGGGGSGGIAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTIT
LASGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTP
EQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 45 – strain ngp165
CSSGGGGSGGGGVAADIGAGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGGKDNSLNTGKLKNDKISRFDFVQKIEVDGQT
ITLASGEFQIYKQDHSAVVALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYTIDFTNKQGYGRIEHLK
TPEQNVELASAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 46 – N-terminal sequence for expression
GPDSDRLQQRRG

SEQ ID NO: 47 – PCR primer
CGCGGATCCCATATGGTCGCCGCCGACATCG

SEQ ID NO: 48 – PCR primer
CCCGCTCGAGTTGCTTGGCGGCAAGGC

SEQ ID NO: 49 – PCR primer
CGCGGATCCCATATGGGCCCTGATTCTGACCGCCTGCAGCAGCGGAGGGTCGCCGCCGACATCGG

SEQ ID NO: 50 – PCR primer
CCCGCTCGAGCTGTTTGCCGGCGATGCC

SEQ ID NO: 51 – PCR primer
CGCGGATCCCATATGGGCCCTGATTCTGACCGCCTGCAGCAGCGGAGGGGAGGGGGTGGTGTCGC

SEQ ID NO: 52 – PCR primer
GCCCAAGCTTCTGTTTGCCGGCGATGCC

SEQ ID NO: 53 – PCR primer
CGCGGATCCCATATGAATCGAACTGCCTTCTGCTGCC

SEQ ID NO: 54 – PCR primer
CCCGCTCGAGTTATTGCTTGGCGGCAAGGC

SEQ ID NO: 55 – PCR primer
GACCTGCCTCATTGATG

SEQ ID NO: 56 – PCR primer
CGGTAAATTATCGTGTTCGGACGGC

SEQ ID NO: 57 – PCR primer
CAAATCGAAGTGGACGGGCAG

SEQ ID NO: 58 – PCR primer
TGTTCGATTTTGCCGTTTCCCTG

SEQ ID NO: 59 – PCR primer
GCTCTAGACCAGCCAGGCGCATAC

SEQ ID NO: 60 – PCR primer
TCCCCCGGGGACGGCATTTTGTTTACAGG

SEQ ID NO: 61 – PCR primer
TCCCCCGGGCGCCAAGCAATAACCATTG

SEQ ID NO: 62 – PCR primer
CCCGCTCGAGCAGCGTATCGAACCATGC

SEQ ID NO: 63 – PCR primer
GCTCTAGATTCTTTCCCAAGAACTCTC

SEQ ID NO: 64 – PCR primer
TCCCCCGGGCCCGTATCATCCACCAC

SEQ ID NO: 65 – PCR primer
TCCCCCGGGATCCACGCAAATACCCC

SEQ ID NO: 66 – PCR primer
CCCGCTCGAGATATAAGTGGAAGACGGA

SEQ ID NO: 67 – Figure 1 sequence
LNQIVK

SEQ ID NO: 68 – Figure 1 sequence
VNRTAFCCLSLTTALILTAC

SEQ ID NO: 69 – Figure 1 sequence
AATTGAACCAAATCGTCAAATAACAGGTTGCCTGTAAACAAAATGCCGTCTGAACCGCCGTTCGGACGACATTTGATTTTTGCTTCTTTGACCTGC
CTCATTGATGCGGTATGCAAAAAAAGATACCATAACCAAAATGTTTATATATTATCTATTCTGCGTATGACTAGGAGTAAACCTGTGAATCGAACT
GCCTTCTGCTGCCTTTCTCTGACCACTGCCCTGATTCTGACCGCCTGC

SEQ ID NO: 70 – Sequence which may be omitted
TRSKP

SEQ ID NO: 71 – Sequence which may be omitted
TRSKPV

SEQ ID NO: 72 – Sequence which may be omitted
PSEPPFG

SEQ ID NO: 73 – Sequence which may be retained
GGGG

SEQ ID NO: 74 – MenB putative Fur box
CATAACCAAAATGTTTATA

SEQ ID NO: 75 – E.coli Fur-box consensus
GATAATGATAATCATTATC

SEQ ID NO: 76 – '936' from MC58, with leader peptide processed
VSAVIGSAAVGAKSAVDRRTTGAQTDDNVMALRIETTARSYLRQNNQTKGYTPQISVVGYNRHLLLLGQVATEGEKQFVGQIARSEQAAEGVYNYI
TVASLPRTAGDIAGDTWNTSKVRATLLGISPATQARVKIVTYGNVTYVMGILTPEEQAQITQKVSTTVGVQKVITLYQNYVQR

SEQ ID NO: 77 – Example hybrid protein
MVSAVIGSAAVGAKSAVDRRTTGAQTDDNVMALRIETTARSYLRQNNQTKGYTPQISVVGYNRHLLLLGQVATEGEKQFVGQIARSEQAAEGVYNY
ITVASLPRTAGDIAGDTWNTSKVRATLLGISPATQARVKIVTYGNVTYVMGILTPEEQAQITQKVSTTVGVQKVITLYQNYVQRGSGGGGVAADIG
TGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTITLASGEFQIYKQNHSAV
VALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTLEQNVELAAAELKADEK
SHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 78 – Sequence for expression
GSGPDSDRLQQRR

SEQ ID NO: 79 – Tandem NMB1870 (MC58 & 2996)
VAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSH
SALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFAAKQGNGKIEHLKSPELNVDLAAADIK
PDGKRHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQGSGPDSDRLQQRRVAADIGAGLADALTAPLDHKDKSLQSL
TLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFL
VSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALF
GDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 80 – NMB1870$_{MC58}$ with N-terminal deletion (ΔG)
VAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSH
SALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFAAKQGNGKIEHLKSPELNVDLAAADIK
PDGKRHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQ

SEQ ID NO: 81 – NMB1870$_{2996}$ with N-terminal deletion (ΔG)
VAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDH
SAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVELAAAELKA
DEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 82 – Tandem NMB1870 (MC58 & 2996)
VAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSH
SALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFAAKQGNGKIEHLKSPELNVDLAAADIK
PDGKRHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQGSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVR
KNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGE
HTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEI
AGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 83 – Tandem NMB1870 (MC58 & M1239)
VAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSH
SALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFAAKQGNGKIEHLKSPELNVDLAAADIK
PDGKRHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQGSGPDSDRLQQRRVAADIGTGLADALTAPLDHKDKGLKSL
TLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKTDSLINQR

SFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTLEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHL
ALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 84 – NMB1870$_{M1239}$ with N-terminal deletion (ΔG)

VAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTITLASGEFQIYK
QNHSAVVALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTLEQNVELAAAE
LKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 85 – Tandem NMB1870 (MC58 & M1239)

VAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSH
SALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFAAKQGNGKIEHLKSPELNVDLAAADIK
PDGKRHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQGSGGGGVAADIGTGLADALTAPLDHKDKGLKSLTLEDSIP
QNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKTDSLINQRSFLVSGL
GGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTLEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRA
QEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 86 – Sequence for expression

GPDSDRLQQRR

SEQ ID NO: 87 – Tandem NMB1870 (2996 & M1239)

GPDSDRLQQRRVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLE
SGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQ
NVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQGSGPDSDRLQQRRVAADIGTGLADALTAPL
DHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNP
DKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTLEQNVELAAAELKADEKSHAVILGDTRY
GSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 88 – Tandem NMB1870 (2996 & M1239)

GPDSDRLQQRRVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLE
SGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQ
NVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQGSGGGGVAADIGTGLADALTAPLDHKDKGL
KSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKTDSLI
NQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTLEQNVELAAAELKADEKSHAVILGDTRYGSEEKGT
YHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 89 – Tandem NMB1870 (M1239 & 2996)

GPDSDRLQQRRVAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTI
TLASGEFQIYKQNHSAVVALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKT
LEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQGSGPDSDRLQQRRVAADIGAGLADALT
APLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNP
DKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRY
GSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 90 – Tandem NMB1870 (M1239 & 2996)

GPDSDRLQQRRVAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTI
TLASGEFQIYKQNHSAVVALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKT
LEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQGSGGGGVAADIGAGLADALTAPLDHKD
KSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLI
NQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGT
YHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 91 – Hybrid of protein '936' and NMB1870$_{2996}$

CVSAVIGSAAVGAKSAVDRRTTGAQTDDNVMALRIETTARSYLRQNNQTKGYTPQISVVGYNRHLLLLGQVATEGEKQFVGQIARSEQAAEGVYNY
ITVASLPRTAGDIAGDTWNTSKVRATLLGISPATQARVKIVTYGNVTYVMGILTPEEQAQITQKVSTTVGVQKVITLYQNYVQRGSGPDSDRLQQR
RVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQD
HSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVELAAAELK
ADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 92 – Hybrid of protein '936' and NMB1870$_{2996}$
CVSAVIGSAAVGAKSAVDRRTTGAQTDDNVMALRIETTARSYLRQNNQTKGYTPQISVVGYNRHLLLLGQVATEGEKQFVGQIARSEQAAEGVYNY ITVASLPRTAGDIAGDTWNTSKVRATLLGISPATQARVKIVTYGNVTYVMGILTPEEQAQITQKVSTTVGVQKVITLYQNYVQRGSGGGGVAADIG AGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVAL QIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVELAAAELKADEKSHA VILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 93 – Hybrid of protein '936' and NMB1870$_{M1239}$
CVSAVIGSAAVGAKSAVDRRTTGAQTDDNVMALRIETTARSYLRQNNQTKGYTPQISVVGYNRHLLLLGQVATEGEKQFVGQIARSEQAAEGVYNY ITVASLPRTAGDIAGDTWNTSKVRATLLGISPATQARVKIVTYGNVTYVMGILTPEEQAQITQKVSTTVGVQKVITLYQNYVQRGSGPDSDRLQQR RVAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTITLASGEFQIY KQNHSAVVALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTLEQNVELAAA ELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 94 – Hybrid of protein '936' and NMB1870$_{M1239}$
CVSAVIGSAAVGAKSAVDRRTTGAQTDDNVMALRIETTARSYLRQNNQTKGYTPQISVVGYNRHLLLLGQVATEGEKQFVGQIARSEQAAEGVYNY ITVASLPRTAGDIAGDTWNTSKVRATLLGISPATQARVKIVTYGNVTYVMGILTPEEQAQITQKVSTTVGVQKVITLYQNYVQRGSGGGGVAADIG TGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTITLASGEFQIYKQNHSAV VALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTLEQNVELAAAELKADEK SHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 95 – oligonucleotide primer
CGCGGATCCGGCCCTGATTCTGACCG

SEQ ID NO: 96 – oligonucleotide primer
CCCGCTCGAGCTGTTTGCCGGCGATGCC

SEQ ID NO: 97 – oligonucleotide primer
CGCGGATCCGGAGGGGGTGGTGTCG

SEQ ID NO: 98 – oligonucleotide primer
CCCGCTCGAGCTGTTTGCCGGCGATGCC

SEQ ID NO: 99 – oligonucleotide primer
CGCGGATCCGGCCCTGATTCTGACCG

SEQ ID NO: 100 – oligonucleotide primer
CCCAAGCTTCTGTTTGCCGGCGATGCC

SEQ ID NO: 101 – oligonucleotide primer
CGCGGATCCGGAGGGGGTGGTGTCG

SEQ ID NO: 102 – oligonucleotide primer
CCCAAGCTTCTGTTTGCCGGCGATGCC

SEQ ID NO: 103 – oligonucleotide primer
CGCGGATCCGGCCCTGATTCTGACCG

SEQ ID NO: 104 – oligonucleotide primer
CCCAAGCTTCTGTTTGCCGGCGATGCC

SEQ ID NO: 105 – oligonucleotide primer
CGCGGATCCGGAGGGGGTGGTGTCG

SEQ ID NO: 106 – oligonucleotide primer
CCCAAGCTTCTGTTTGCCGGCGATGCC

SEQ ID NO: 107 – oligonucleotide primer
CGCGGATCCGGCCCTGATTCTGACCG

SEQ ID NO: 108 – oligonucleotide primer
CCCGCTCGAGCTGTTTGCCGGCGATGCC

SEQ ID NO: 109 – oligonucleotide primer
CGCGGATCCGGAGGGGGTGGTGTCG

SEQ ID NO: 110 – oligonucleotide primer
CCCGCTCGAGCTGTTTGCCGGCGATGCC

SEQ ID NO: 111 – oligonucleotide primer
CGCGGATCCGGCCCTGATTCTGACCG

SEQ ID NO: 112 – oligonucleotide primer
CCCGCTCGAGCTGTTTGCCGGCGATGCC

SEQ ID NO: 113 – oligonucleotide primer
CGCGGATCCGGAGGGGGTGGTGTCG

SEQ ID NO: 114 – oligonucleotide primer
CCCGCTCGAGCTGTTTGCCGGCGATGCC

SEQ ID NO: 115 – oligonucleotide primer
CGCGGATCCGGCCCTGATTCTGACCG

SEQ ID NO: 116 – oligonucleotide primer
CCCAAGCTTCTGTTTGCCGGCGATGCC

SEQ ID NO: 117 – oligonucleotide primer
CGCGGATCCGGAGGGGGTGGTGTCG

SEQ ID NO: 118 – oligonucleotide primer
CCCAAGCTTCTGTTTGCCGGCGATGCC

SEQ ID NO: 119 – oligonucleotide primer
CGCGGATCCCATATGGGCCCTGATTCTGACCG

SEQ ID NO: 120 – oligonucleotide primer
CGCGGATCCCTGTTTGCCGGCGATGCC

SEQ ID NO: 121 – oligonucleotide primer
CGCGGATCCCATATGGGCCCTGATTCTGACCG

SEQ ID NO: 122 – oligonucleotide primer
CGCGGATCCCTGTTTGCCGGCGATGCC

SEQ ID NO: 123 – strain FN131217
MNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDASGKLTYTIDFA
AKQGHGKIEHLKSLELNVDLAASDIKPDKKRHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGSAEVETANGIRHIGLAAKQ

SEQ ID NO: 124 – strain ES14933
MNRTAFCCFSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQIYKQDHSALTALQTEQEQDPEHSGKMVAKRRFKIGDIAGEHTSFDKLPKDVMATYRGTAFGSDDAGGKLTYTIDFA
AKQGHGKIEHLKSPELNVELATAYIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 125 – strain GB0993
MNRTAFCCLSLTTALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLMLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKDVMATYRGTAFGSDDAGGKLTYTIDFA
AKQGHGKIEHLKSPELNVELAAAYIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIQHIGLAAKQ

SEQ ID NO: 126 – strain M6190
MNRTTFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVNGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSRKMVAKRQFRIGDIAGEHTSFDKLPKGDSATYRGTAFGSDDAGGKLTYTIDFA
AKQGYGKIEHLKSPELNVDLAAAYIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVKTANGIRHIGLAAKQ

SEQ ID NO: 127 – strain F19324

MNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQVYKQSHSALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFA
AKQGNGKIEHLKSPELNVDLAAADIKPDGKRHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQ

SEQ ID NO: 128 – strain ISS1113

MNRTAFCCLSLTTALILTACSSGGGGVTADIGTGLADALTAPLDHKDKGLKSLTLEDSISQNGTLTLSAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGKLITLESGEFQVYKQSHSALTALQTEQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKGGSATYRGTAFGSDDAGGKLTYTIDFA
AKQGHGKIEHLKSPELNVELATAYIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 129 – strain gb0345

MNRTAFCCFSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGKLITLESGEFQVYKQSHSALTALQTEQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKGGSATYRGTAFGSDDAGGKLTYTIDFA
AKQGHGKIEHLKSPELNVELATAYIKPDEKRHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIHHIGLAAKQ

SEQ ID NO: 130 – strain M0445

MNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKGGSATYRGTAFSSDDAGGKLTYTIDFA
AKQGHGKIEHLKSPELNVELATAYIKPDEKRHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIQHIGLAAKQ

SEQ ID NO: 131 – strain MK82

MNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPVDKAEYHGKAFSSDDAGGKLTYTIDFAA
KQGHGKIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 132 – strain 8047

MNRTAFCCLSLTAALILTACSSGGGGVAADIGARLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAA
KQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 133 – strain C4678

MNRTAFCCLSLTTALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDAGGKLTYTIDFAA
KQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 134 – strain ISS1133

MNRTAFCCLSLTTALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAA
KQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 135 – strain NG6/88

MNRTAFCCLSLTTALILTACSSGGGGVAADIGTGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPSGKAEYHGKAFSSDDAGGKLTYTIDFAA
KQGHGKIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 136 – strain M0579

MNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPSGKAEYHGKAFSFDDAGGKLTYTIDFAA
KQGHGKIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 137 – strain F16325

MNRTAFCCFSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPSGKAEYHGKAFSSDDPNGRLHYSIDFTK
KQGYGRIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID NO: 138 – strain gb988

MNRTTFCCLSLTAALILTACSSGGGGSGGGGVAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLK
NDKISRFDFVQKIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPGDKAEYHGKAFSSDDPNGRL
HYTIDFTNKQGYGRIEHLKTPELNVDLASAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID NO: 139 – strain 220173i

MNRTAFCCLSLTTALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFD
FIRQIEVDGQLITLESGEFQVYKQSHSALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFA
AKQGNGKIEHLKSPELNVDLAAADIKPDGKRHAVISGSVLYNQAEKGSYSLGIFGGKA

SEQ ID NO: 140 – strain gb101

MNRTTFCCLSLTAALILTACSSGGGGSGGGGVAADIGAGLADALTAPLDHKDKGLKSLTLEDSISQNGTLTLSAQGAERTFKAGDKDNSLNTGKLK
NDKISRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIVGEHTSFGKLPKDVMATYRGTAFGSDDAGGK
LTYTIDFAAKQGHGKIEHLKSPELNVDLAAADIKPDEKHHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGSAEVETANGIRHIGLAAKQ

SEQ ID NO: 141 – strain nge31

MNRTAFCCLSLTAALILTACSSGSGGGGVAADIGTGLAYALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSR
FDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSIDF
TKKQGYGRIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHEIGIAGKQ

SEQ ID 142 – Triple NMB1870 tandem (MC58, 2996 and m1239)

VAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSH
SALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFAAKQGNGKIEHLKSPELNVDLAAADIK
PDGKRHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQGSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVR
KNEKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGE
HTAFNQLPDGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEI
AGSATVKIGEKVHEIGIAGKQGSGGGGVAADIGTGLADALTAPLDHKDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKI
SRFDFVQKIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLPGGKAEYHGKAFSSDDPNGRLHYSI
DFTKKQGYGRIEHLKTLEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

SEQ ID 143 – NadA from Haji strains

MKHFPSKVLTTAILATFCSGALAATNDDDVKKAATVAIAAAYNNGQEINGFKAGETIYDIDEDGTITKKDATAADVEADDFKGLGLKKVVTNLTKT
VNENKQNVDAKVKAAESEIEKLTTKLADTDAALADTDAALDATTNALNKLGENITTFAEETKTNIVKIDEKLEAVADTVDKHAEAFNDIADSLDET
NTKADEAVKTANEAKQTAEETKQNVDAKVKAAETAAGKAEAAAGTANTAADKAEAVAAKVTDIKADIATNKDNIAKKANSADVYTREESDSKFVRI
DGLNATTEKLDTRLASAEKSITEHGTRLNGLDRTVSDLRKETRQGLAEQAALSGLFQPYNVGRFNVTAAVGGYKSESAVAIGTGFRFTENFAAKAG
VAVGTSSGSSAAYHVGVNYEW

SEQ ID NO: 144 – glycine linker

GSGGGG